Molecular Genetic Analysis of Light Signalling Pathways in Tomato

Thesis

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Molecular Genetic Analysis of Light Signalling Pathways in Tomato

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Contents

List of Abbreviations

A535	absorbance at 535 nm
AC	Ailsa Craig genotype of tomato
Af	*Anthocyanin-fruit* mutant of tomato
Atv	*atroviolacea* mutant of tomato
B	blue light
CAB	gene encoding chlorophyll *a/b* binding protein
CHS1	gene encoding chalcone synthase
Cop	constitutive photomorphogenic mutant of *Arabidopsis*
CRY	gene encoding cryptochrome
D	dark
DDB1	UV-damaged DNA binding protein 1
det1	de-etiolated 1 mutant of *Arabidopsis*
dg	*dark-green* mutant of tomato
EMS	ethyl methane sulphonate
FMN	flavin mono nucleotide
FR	far red light
fri	far red light-insensitive mutant of tomato
fr wt	fresh weight
fus	*fusca* mutants of *Arabidopsis*
GFP	green fluorescent protein
GUS	β-glucuronidase
HIR	high irradiance response
hp	*high pigment* mutant of tomato
hy	long-hypocotyl mutant of *Arabidopsis*
LFR	low fluence response
MM	Money Maker genotypes of tomato
NDPK	nucleoside diphosphate kinase
nos	gene encoding neomycin phosphotransferase II
PAS	Per/Arnt/Sim protein domain
PHOT1	gene encoding phototropin 1
PHY	gene encoding phytochrome
PHY	Immunochemically detectable product of *phy* gene
PIF	phytochrome interacting factor
PKS1	phytochrome kinase substrate 1
Pfr	far-red light-absorbing form of phytochrome
Pr	red light-absorbing form of phytochrome
PTGS	post transcriptional gene silencing
R	red light
siRNA	short interfering RNA
TDET1	product of tomato *DET1* gene
T-DNA	transfer DNA of *Agrobacterium*
tri	temporarily red light insensitive mutant of tomato
UV	ultraviolet light
VLFR	very low fluence response
WA	Walter genotype of tomato
WT	wild type
WL	white light

Abstract

The research undertaken in this thesis is focused on the study of photomorphogenesis in tomato (*Solanum lycopersicon*). It includes a detailed physiological characterization of several previously described but largely understudied mutants that display light hyperresponsive phenotypes, as well as a reverse genetic study focused on the photomorphogenesis regulator DE-ETIOLATED1 (DET1). A range of light-hyperresponsive mutants were selected in order to gain new insights into light signal transduction, including *atroviolacea* (*atv*), *Anthocyanin fruit* (*Af*), *Punctate* (*Pn*) and *dark green* (*dg*), which were studied alongside the better characterized *high pigment* (*hp1* and *hp2*) mutants. Studies of these mutants are presented with an emphasis on anthocyanin pigment biosynthesis and distribution, chlorophyll accumulation, plastid development, and light regulated gene expression in different light conditions. Other work in the thesis has focused on *DET1*, mutations in which are responsible for the tomato *hp2* mutation. To better understand the function and importance of DET1 in tomato, several transgenic lines were generated containing different *DET1* gene constructs. Unexpectedly, phenotypes characteristic of DET1 suppression were observed, e.g., dwarfness and higher carotenoid accumulation in fruits, and molecular analysis indicated in all cases that these phenotypes were a result of suppression of endogenous *DET1* expression caused by post-transcriptional gene silencing (PTGS). In an attempt to harness the biotechnological potential of *DET1* gene suppression in fruits and to avoid the collateral negative effects on plant growth, expression of the *DET1* gene was then modulated only within the fruits using fruit-specific promoters. The results show that suppression of *DET1* expression specifically in the fruits enhances carotenoid content but does not affect plant growth and development, and fruit-specific *DET1* gene silencing was confirmed at the molecular level. These results provide a novel example of the use of tissue-specific gene silencing to improve the nutritional value of plant-derived products.

CHAPTER 1

General Introduction

1.1 Photomorphogenesis

Some organisms can cope with changing environmental conditions by their capacity to move, whereas sessile plants can only acclimate to changes by altering their physiology and growth pattern. Light has long been known to be an important signal for plant development, influencing nearly all aspects of the life cycle. Plants use light in two ways: they convert light energy to chemical energy via photosynthesis and they use light as a source of information for evaluating some of the properties of the local environment. Plants accurately monitor fluctuations in the intensity, spectral quality, directionality and periodicity (day length) of incoming light. The remarkable influence that light has on the growth of plants is clearly visible during seedling development. Dark-grown seedlings display an etiolated phenotype characterized by closed apical hooks, unexpanded cotyledons containing etioplasts, and elongated hypocotyls. This developmental programme (known as skotomorphogenesis) is an adaptation of the developing seedling to grow through soil to reach the light. Upon exposure to light, seedlings undergo a series of developmental changes known as de-etiolation, involving opening of the apical hook, expansion of cotyledons, which then begin to photosynthesize, inhibition of hypocotyl elongation, and the initiation of cell differentiation in vegetative meristems. These events are known as photomorphogenesis (Figure 1.1). More than 80 years ago Garner and Allard (1920) demonstrated that the pigments necessary for such photomorphogenic responses were distinct from the pigments required for photosynthesis. These early photobiological experiments led to the discovery of the phytochromes, arguably the most important plant photoreceptors.

1.2 Plant Photoreceptors

The first step in the network that connects the genetic developmental programme to environmental cues involves the perception of light by photoreceptors. There are three known classes of photoreceptors in higher plants, red/far red light-absorbing phytochromes (Fankhauser and Staiger, 2002), blue/UV-A light-absorbing cryptochromes (Cashmore et al., 1999) and blue/UV-A-absorbing phototropins (Briggs and Christie, 2002). These photoreceptors can monitor the presence, absence, spectral quality (wavelength), fluence rate (intensity), direction and diurnal duration of the incident light. The phytochromes and the cryptochromes control growth and developmental responses, whereas the phototropins function primarily in controlling directional (phototropic) growth.

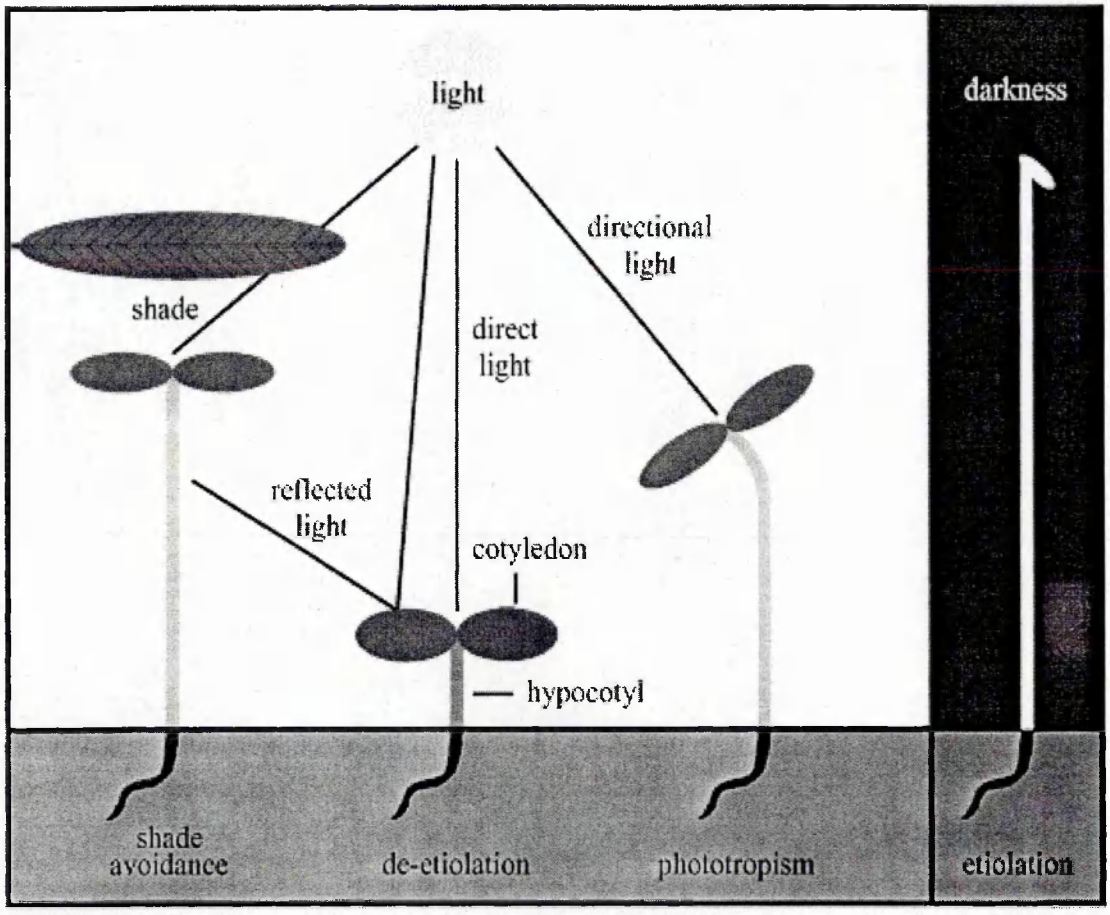


Figure 1.1. Schematic representation of photomorphogenic developmental patterns of tomato seedlings grown in different light environments. After McNellis and Deng (1995).

1.2.1 Phototropins

The blue region of the visible spectrum primarily induces light-driven tropic growth in mature plants. In *Arabidopsis* two photoreceptors, phot1 and phot2 have been identified for this specific light response (Briggs and Christie, 2002). Phot1 and phot2 are Ser/Thr-protein kinases with amino-terminal chromophore-binding domains. Two FMN molecules bind to LOV (light oxygen voltage) domains. LOV domains are a subset of PAS (Per/Arnt/Sim) domains that are often found in proteins that sense environmental signals. Phot1 has been found to associate with the plasma membrane, whereas the localization of phot2 is currently unknown (Briggs and Christie, 2002). Both phot1 and phot2 function in phototropic hypocotyl growth and are also essential for other blue light responses. Their role has been demonstrated for the inhibition of hypocotyl growth during the very first minute of blue light irradiation, for light-regulated ion fluxes, for chloroplast movement and for stomatal opening (Folta and Spalding, 2001; Kinoshita et al., 2001; Sakai et al., 2001). For stomatal opening they have redundant functions, whereas for positive phototropism of the hypocotyl phot1 clearly plays the predominant role in low light intensities, and both phot1 and phot2 have redundant functions in high light intensities (Kinoshita et al., 2001; Sakai et al., 2001). Not much is known about the downstream regulators of these photoreceptors.

1.2.2 Cryptochromes

The first blue light photoreceptor to be identified was a cryptochrome, through the isolation of a T-DNA-tagged allele of the *hy4* mutant of *Arabidopsis* (Ahmad and Cashmore, 1993). Cryptochromes show sequence similarity to photolyases, a family of proteins that mediate repair of UV-damaged DNA. Photolyases are a rare class of flavoproteins that mediate a redox reaction in response to the absorption of light (Sancar, 1994). Whereas cryptochromes are flavoproteins, they lack photolyase activity (Lin et al., 1995) and are commonly (although not always) characterized by a distinguishing C-terminal domain that is not present in photolyases (Cashmore et al., 1999).

In *Arabidopsis* there are two well characterized cryptochromes, cry1 and cry2. Both have important functions during de-etiolation and for the transition from vegetative to reproductive growth (Lin, 2000; Quail, 2002b). During de-etiolation, cry2 is particularly important in

response to low blue light intensities, in contrast to cry1, which has a prevalent role in response to strong blue light (Lin, 2000). Interestingly, in contrast to cry2, which is strongly down-regulated in response to blue light, cry1 protein levels are not light-regulated. This explains why cry2 plays a major role in low light whereas cry1 is more important in high light (Cashmore et al., 1999; Lin, 2000; Quail, 2002b). Recently a third cryptochrome has been cloned from *Arabidopsis,* which is closely related to *Synechocystis* cryptochrome and is targeted to organelles (Kleine et al., 2003). The GFP fusion proteins with either full-length or N-terminal fragments of *CRY3* showed that they were translocated into both chloroplasts and mitochondria. However the detailed function of this third cryptochrome has yet to be characterized.

Cryptochromes are found in the nucleus of *Arabidopsis* cells, both in the dark and in the light (Cashmore et al., 1999; Kleiner et al., 1999). However, a fusion protein between β-glucuronidase (GUS) and the carboxy-terminal domain of cry1 is cytosolic in the light and nuclear in the dark, raising the possibility for action in both subcellular compartments (Yang et al., 2001). Recent experiments strongly suggest that expression of the C-terminus of either *Arabidopsis CRY1* or *CRY2* confers a striking phenotype on dark-grown seedlings. This phenotype is very similar to the constitutive light responses exhibited by many *cop/det/fus* mutants (see below) (Yang et al., 2001). This de-etiolation response appears to require the physical interaction between the carboxy-terminal domain of the cryptochromes and the COP1 protein (see below) (Wang et al., 2001; Yang et al., 2001).

1.2.3 Phytochromes

Phytochromes are the best-characterized plant photoreceptors, both biochemically and physiologically. Phytochromes are typically encoded by small multigene families in all plants examined, e.g., *PHYA-PHYE* in *Arabidopsis* (Møller et al., 2002; Quail 2002[a]); Nagy and Schafer, 2002) and *PHYA*, *PHYB1*, *PHYB2*, *PHYC* and *PHYE* in tomato (Pratt et al., 1997). In *Arabidopsis*, *PHYA, B, C* and *E* are evolutionarily divergent proteins, sharing only 46% to 53% sequence identity, whereas *PHYD* encodes an apoprotein that shares 80% sequence identity with *PHYB* (Clack et al., 1994).

Phytochromes are soluble dimeric chromoproteins that consist of two ~125 kDa polypeptides. Each polypeptide folds into two main structural domains, an amino-terminal photosensory domain that cradles a single, covalently-attached tetrapyrrole chromophore, typically phytochromobilin, and a carboxy-terminal domain that mediates dimerization and probably also signal transduction (Quail, 2002[b]). Each phytochrome can exist in two photointerconvertible conformations, a red light-absorbing Pr form and a far/red light-absorbing Pfr form. In the dark phytochrome is synthesized in the Pr form, which is biologically inactive. Upon absorption of red light phytochrome is photoconverted into the active Pfr form. Because sunlight is enriched in red light (compared to far/red light), phytochrome is predominantly found in the Pfr form in the light. Pfr formation initiates an intracellular transduction process culminating in altered expression of selected genes that are responsible for photomorphogenesis, whereas reconversion to Pr can abrogate this process.

Daylight contains roughly equal proportions of red and far/red light (red:far red ≈1.2), but within dense vegetation this ratio is lowered by the absorption of red light by photosynthetic pigments. Changes in the red:far/red light ratio is a more reliable indicator of the proximity of potentially competing neighbours than is the concomitant reduction in the total amount of light penetrating the canopy. Plants therefore use the phytochromes as proximity sensors to modify their growth and development, constituting the 'shade-avoidance syndrome' (Smith 1995).

Phytochrome responses have been defined by their wavelength and fluence-rate (intensity) requirements into three groups: very low fluence responses (VLFR), low fluence responses (LFR) and high irradiance responses (HIR). The classical example of a phytochrome-mediated LFR (fluence requirement 1–1000 μmol m^{-2}) is the red light-induced germination of lettuce seeds. This induction can be inhibited by subsequent far/red light treatment. Seeds can be repeatedly treated by sequential red or far/red light, and the ultimate germination response depends only on the last light treatment. Thus, photoreversibility is one characteristic feature of LFR responses (Neff et al., 2000).

PhyA is unique among all phytochromes because it alone is responsible for the very-low-fluence response (VLFR) and for the far/red light-dependent high-irradiance response (HIR). The VLFR includes light effects on the expression of some genes, seed germination and the

gravitotropic control of hypocotyl growth, and it can be induced with extremely low photon fluences of 0.001–1.000 μmol $m^{-2}$of either red or far/red light pulses. The HIR requires relatively high photon fluence rates and a long duration of irradiation, and it is fluence rate and not total fluence that defines this type of response. Typical HIRs include inhibition of hypocotyl elongation, opening of the apical hook, expansion of the cotyledons, accumulation of anthocyanin, and a far/red light preconditioned block of greening during seedling development (Barnes et al., 1996; Neff et al., 2000). The central dogma for phytochrome action (that Pfr is the biologically active form) applies to the LFRs mediated by phyB–phyE and the VLFRs mediated by phyA. However, for phyA-mediated far/red light HIRs, a short-lived intermediate generated during Pfr-to-Pr photoconversion has been suggested to be the physiologically active form (Shinomura et al., 2000).

1.3 Phytochrome Localization

The phytochrome apoproteins are synthesized within the cytosol and for years phytochromes were considered to be entirely cytosolic, but now there is strong evidence for photoactivated nuclear translocation of phytochromes (Nagy and Schafer, 2002). Both phyA and phyB tagged with green fluorescent protein (GFP) show light-activated import into the nuclei of tobacco (Kircher et al., 1999) and *Arabidopsis* cells (Yamaguchi et al., 1999). Import of phyB occurs only in the Pfr form and is slow, requiring several hours for full mobilization. Phytochrome A either as Pfr_A, or as Pr_A that has been photoconverted through Pfr_A and back again, moves more rapidly (about 15 min).

The photobiological criteria for functionality are satisfied because phyB transport is activated by red light irradiation and inhibited by far red light irradiation, whereas the transport of phyA is maximal under continuous far/red light irradiation. These facts provide a framework for understanding the phytochrome regulation of gene expression through the translocation of Pfr into the nucleus, interaction with primary reaction partners and direct regulation of the promoters of light-regulated genes (see later). These and other findings suggest that phytochrome may activate signalling pathways in both the cytoplasm and the nucleus.

1.3.1 Phytochrome Signalling in the Cytoplasm

The evidence for cytoplasmically-localized phytochrome signalling events comes from biochemical and pharmacological studies that have implicated the involvement of G-proteins, cGMP, calcium and calmodulin in the control of phytochrome-dependent gene expression (Shacklock et al., 1992; Bowler et al., 1994). Reverse genetics approaches have subsequently provided further support for the involvement of G-proteins. One such example is that *Arabidopsis* plants ectopically overexpressing the alpha subunit of the heterotrimeric G-protein, regardless of the G_{α} activation state, were hypersensitive to red and far/red light (Okamoto et al., 2001[a]). However, a recent report by Jones et al. (2003) indicates that loss-of-function of the single-copy genes encoding canonical G_{α} and G_{β} subunits did not result in altered red and far/red light sensitivities.

These results therefore do not support that the phytochrome control of seedling photomorphogenesis involves a heterotrimeric G protein. Nonetheless, a role for calcium in light signalling has been reinforced by the identification of SUB1, a cytoplasmically-localized calcium-binding protein that appears to negatively regulate cryptochrome and phyA responses (Guo et al., 2001).

1.4 Phytochrome Signal Transduction

The most extensive advances in understanding phytochrome signal transduction have come from studies of photomorphogenic mutants (Kendrick et al., 1997). The initial screens for photomorphogenic mutants, which led to the identification of the first photoreceptor mutants in *Arabidopsis*, were performed in white light. However, in later years, broadband red (R), far/red (FR) and blue (B) light were used to identify mutants deficient in photoreceptors as well as signal transduction intermediates. Genetic screens have identified numerous loci that are involved in phyA and phyB signalling processes. The genetic evidence suggest that there are both shared and separate early signalling pathways used by phyA and phyB, and that there are both positively and negatively acting components in each pathway (Fankhauser, 2001;

Quail, 2002[a]). Mutations in specific phytochrome genes allow the analysis of the physiological functions of the different phytochromes.

Many putative light signal transduction intermediates have been identified from mutant screens aimed principally at isolating mutants insensitive to light or mutants displaying constitutive photomorphogenesis in darkness (Møller et al., 2002). The most severe white light-insensitive mutants include photoreceptor mutants, as well as one mutated in a gene encoding a bZIP transcription factor known as HY5 (Oyama et al., 1997). The severity of this mutant demonstrates that HY5 plays a key role, most likely as a positive regulator, in the control of photomorphogenesis. While *hy5* mutants are affected in both phyA and phyB signalling, the majority of the mutants identified so far are specific to phyA.

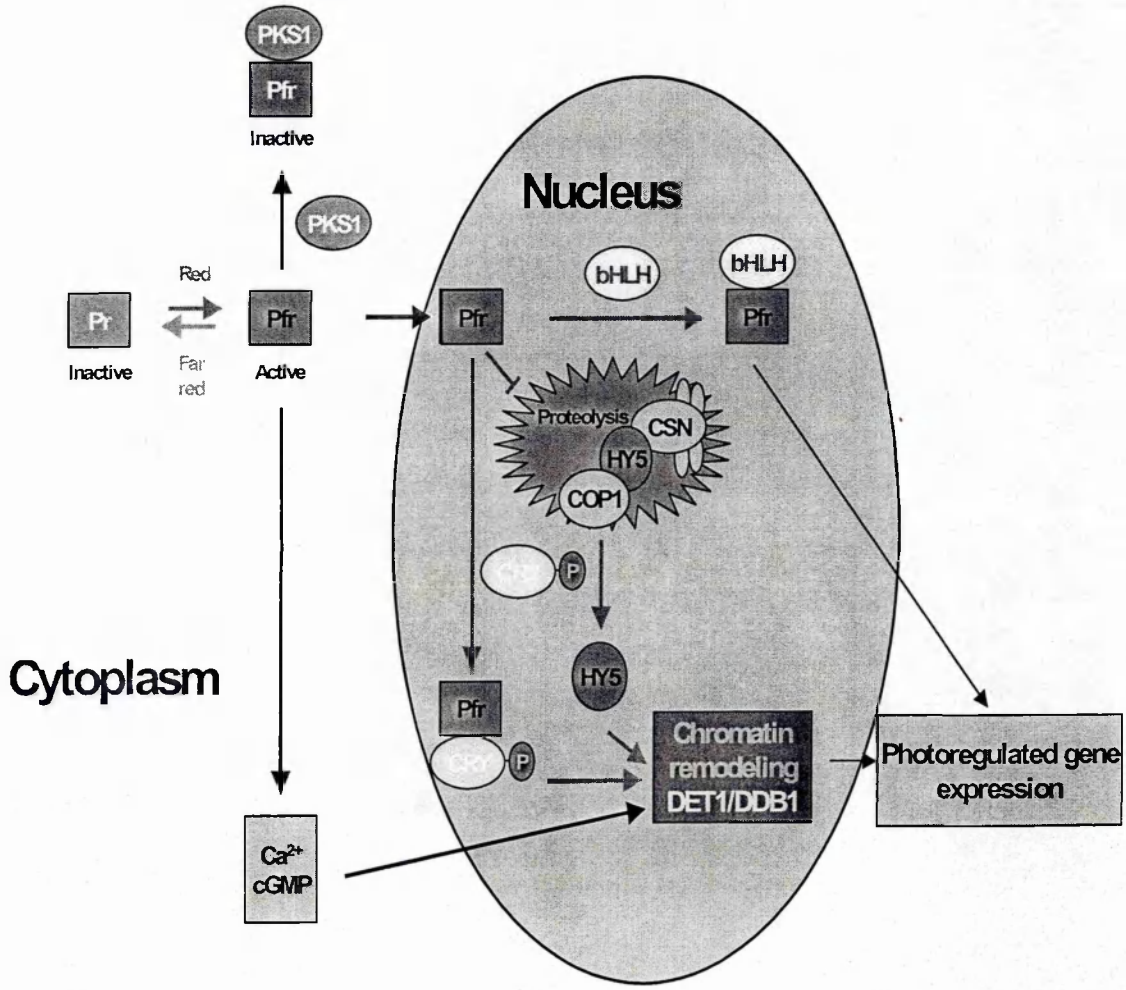


Figure 1.2. Schamatic representation of phytochrome signalling in *Arabidopsis*

Mutants for several components affected in the HIR branch of the phyA signalling pathway have been identified, including *fhy1*, *fhy3* (Whitelam et al., 1993), *fin2* (Soh et al., 1998), *spa1* (Hoecker et al., 1998), *far*1 (Hudson et al., 1999), *fin*219 (Hsieh et al., 2000), *pat*1 (Bolle at al., 2000), *eid1* (Buche et al., 2000), *laf1* (Ballesteros et al., 2001), *laf6* (Møller et al., 2001) and *fhy4* (Fry et al., 2002). The *fhy1*, *fhy3*, *fhy4*, *fin2*, *fin219*, *far1*, *laf1* and *laf6* mutants show reduced sensitivity to continuous far red light, indicating that these genes encode positive regulators of phyA signalling pathways (Wang et al., 2003).

Some of the phyA signalling intermediates have been characterized at the molecular level whereas others await molecular identification. LAF6 is a plastid-localized ATP-binding protein involved in coordinating intercompartmental communication between the plastid and the nucleus (Møller et al., 2001). PAT1 is a new member of the GRAS (*GAI*, *RGA*, *SCR*) family (Bolle et al., 2000), whereas FIN219 is a GH3-like protein whose expression is rapidly induced by auxin (Hsieh et al., 2000). Both PAT1 and FIN219 are cytoplasmic proteins, whereas FAR1, FHY3, SPA1, HFR1, LAF1 and EID1 are all nuclear. FHY1 is a novel protein (Desnos et al., 2001) whereas HFR1 is an atypical basic helix–loop–helix (bHLH) transcription factor closely related to PIF3, and LAF1 is a Myb-type transcriptional activator (Wang et al., 2003).

Only two mutants (*spa1* and *eid1*) show enhanced phyA-specific light responses. Genetic and physiological results indicate that the encoded proteins function as negatively acting components of phyA signalling and are involved in different, but interacting, phyA-dependent signal transduction chains. SPA1 functions as a negative regulator in both the VLFR and the HIR, whereas EID1 is only involved in signalling cascades regulating the HIR (Zhou et al., 2002). SPA1 contains WD-40 repeats similar to COP1 and exhibits some weak homology with protein kinases (Hoecker et al., 1999), whereas EID1 is an F-box protein and a likely component of so called SCF (Skp1/Cdc53/F-box protein) complexes (Dieterle et al., 2001). The biochemical function of most known phyA signalling molecules (such as PAT1, FAR1, FHY3 and FIN 219) remains largely unknown and only recently have SPA1 and LAF1 functions been investigated more thoroughly (see below).

One large class of *Arabidopsis* mutants designated constitutive photomorphogenic or de-etiolated mutants, are defective in genes encoding the COP/DET/FUS family of proteins.

Eleven loci have been found to be associated with this group (*det1, cop1, cop8, cop9, cop10, cop11, cop16, fus5, fus8, fus11* and *fus12*) (Hardtke and Deng, 2000). Because these mutations are recessive and pleiotropic, these gene products are generally considered to act negatively in darkness to suppress photomorphogenesis, and to act late in the signalling process downstream of both the phy and cry pathways. Some of these are now quite well characterized including COP9 signalosome components, COP1, COP10, HY5 and DET1.

Recent findings indicate that one of these proteins, CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), functions as an E3 ubiquitin-protein ligase, targeting specific proteins for degradation by assisting in their ubiquitination (Hardtke and Deng, 2000; Osterlund et al., 1999; Schwechheimer et al., 2001; Seo et. al., 2003). Other proteins are subunits of the COP9 signalosome complex that is closely related to the lid sub-complex of the 26S proteasome, known to be responsible for degradation of ubiquitinated proteins (Schwechheimer et al., 2000; Wei et al., 1999). HY5 is a constitutively nuclear bZIP transcription factor that is well documented to function positively in phytochrome signalling by binding to the promoters of light-inducible genes (Osterlund et al., 2000).

Levels of HY5 protein remain low in darkness, but increase in the light. Current evidence indicates that COP1 interacts directly with HY5 in the nucleus, potentially facilitating its ubiquitination, and hence specifically targeting it for proteasome-mediated degradation in the dark. Exposure to light reduces the nuclear abundance of COP1, thereby reducing the rate of HY5 protein degradation and allowing HY5 to accumulate. Evidence has been presented that the light-induced change in COP1 nuclear abundance is mediated by either phyA, phyB or cry1, consistent with the placement of COP1 downstream of these pathways (Osterlund et al., 1999). The mechanism by which this occurs is unclear, but recent evidence for the physical interaction of COP1 with cry1, cry2 and the carboxy-terminal domain of phyB might indicate a very direct pathway (Wang et al., 2001; Yang et al., 2001).

Additional support for the proposed role of proteasome-mediated protein degradation in phytochrome signalling has come from studies on two other loci, *EMPFINDLICHER IM DUNKELROTEN LICHT 1* (*EID1*) and *SUPPRESSOR OF PHYA* (*SPA1*), which were genetically identified as specific negative regulators of phyA signalling. EID1 has been identified as a new, nuclear-localized F-box protein, that seems likely to function as a

substrate-specific subunit of an SCF-type E3 ubiquitin ligase (Okamoto et al., 2001[b]). SPA1 is a nuclear-localized, WD-40-repeat-containing protein that has high sequence similarity to COP1 in its WD-40 domain and which binds strongly to COP1 (Hoecker et al., 1999). SPA1 has no effect on COP1 auto-ubiquitination, but facilitates LAF1 ubiquitination by COP1 at low COP1 concentrations. These results indicate that in darkness COP1 acts as a repressor by promoting the ubiquitin-mediated proteolysis of a subset of positive regulators. Following activation of phyA, SPA1 stimulates the E3 activity of residual nuclear COP1 to ubiquitinate LAF1, thereby desensitizing phyA signals (Seo et. al., 2003).

Mutations in *DE-ETIOLATED 1* (*DET1*) have led to the discovery of another mechanism of regulation in phytochrome signal transduction. Mutations in *DET1* are known to result in constitutive de-etiolation in the dark, like *cop/fus* mutants, implying that DET1 also plays an important role in the repression of light-induced genes (Quail 2002[b]). DET1 is not a part of the COP9 signalosome and does not have a proteolysis-related activity, indicating that it does not participate in the regulation of proteolysis. Recent findings suggest that DET1 binds to nucleosome core particles via an interaction with the N-terminal tail of histone H2B (Benvenuto et al., 2002). Furthermore, DET1 is also part of a complex with UV-DDB1, which in animal cells is part of histone acetyltransferase complexes (Schroeder et al., 2002). The significance of these findings is reinforced by the observation that DET1 binds preferentially to non-acetylated H2B tails (Benvenuto et al., 2002), suggesting that it may bind to the nucleosomes around light-inducible genes and prevent gene expression in the dark. The phenotypes of the *cop/det/fus* mutants suggest that chromatin remodelling is equally important in controlling light-dependent gene expression as protein degradation, although the links between the two processes have yet to be identified. The role of DET1 during development in tomato has been examined in Chapter 3.

1.5 Phytochrome-Interacting Factors

Yeast two-hybrid screens have identified phytochrome-interacting factors as potential primary signalling partners (Ni et al., 1998; Fankhauser et al., 1999). One protein identified in these screens was PHYTOCHROME-INTERACTING FACTOR 3 (PIF3), a member of the basic helix–loop–helix (bHLH) superfamily of transcription factors. Subsequent *in vitro* interaction assays showed that full-length, chromophore-conjugated molecules of both phyA and phyB

bind to PIF3, but only after light-induced conversion to the biologically-active Pfr form (Ni et al., 1999).

PIF3 localizes constitutively to the nucleus and binds in a sequence-specific fashion to a G-box DNA sequence, CACGTG, that is present in various light-regulated promoters (Martinez-Garcia et al., 2000). Moreover, phyB can bind specifically and photoreversibly to PIF3 that is already bound to its cognate DNA-binding site. Together with the observed light-induced translocation of phy molecules to the nucleus, these data indicate that PIF3 could recruit the photoreceptor in its active form to G-box-containing promoters. RNA-blot analysis of PIF3-deficient seedlings indicates that PIF3 is functionally necessary for phyB-induced expression of two key photoresponsive genes, *CIRCADIAN CLOCK-ASSOCIATED PROTEIN 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) (Quail, 2002[b]).

Other phytochrome interacting partners have been identified from studies involving yeast two-hybrid screening. One of these is denoted phytochrome kinase substrate 1 (PKS1), a cytosolic protein that accepts a phosphate group from phyA (Fankhauser et al., 1999). Transgenic expression showed that PKS1 is a negative regulator of photomorphogenesis specific to phyB. PhyA also interacts with a nucleoside diphosphate kinase (NDPK2), found in both the cytoplasm and the nucleus (Choi et al., 1999). The kinase activity of NDPK2 is increased about two-fold when bound to recombinant phyA in the Pfr form, although an in vitro activation of NDPK2 by red or far/red irradiation has not been reported. The regulation of gene expression could, in principle, emanate from kinase activity of phytochrome per se, and/or activation of NDPK2 (Smith, 2000). Present day knowledge about phytochrome signalling in *Arabidopsis* has been represented in Figure 1.2.

1.6 Light Signalling in Tomato

Tomato (*Solanum lycopersicon*) has become a model species complementary to *Arabidopsis* in which to characterize light signalling, thus enabling more general conclusions to be drawn about the roles played in higher plants by individual photoreceptors (Kendrick *et al.* 1997; Pratt *et al.* 1997). In addition, studies in tomato can reveal the roles of photoreceptors in the control of fruit ripening. There are several photomorphogenic mutants in tomato related to phytochrome function (Kendrick et al., 1994). Genetic, spectrophotometric, immunochemical

and physiological investigations of two far/red-light-insensitive (*fri*) mutants isolated following ethyl methane sulphonate (EMS) treatment of tomato seeds have led to the hypothesis that they are deficient in phyA (Van Tuinen *et al.* 1995). Moreover, genetic mapping of the *FRI* locus and RFLP mapping of *PHYA* (the gene encoding PHYA, the apoprotein of phyA) indicate that they are at the same location on tomato chromosome 10 (Van Tuinen *et al.* 1997).

Recently several tomato mutants at the locus *tri* (temporarily red light insensitive) have been isolated, which have a reduced inhibition of hypocotyl growth specifically in red light (Lazarova et al., 1998). In these mutants, this lack of inhibition is only temporary and lasts for 2 days upon transfer of etiolated seedlings to red light. A study of anthocyanin biosynthesis in the *tri* mutants revealed that at very low red fluence rates a similar level to wild-type was found, whereas at high fluence rates of red light the level was severely reduced. While the levels of phyA have been shown to be normal in this mutant, recently it has been shown that it lacks a light-stable phytochrome (Lazarova et al., 1998).

Tomato in fact contains five phytochromes, including homologues of *Arabidopsis* phyA, C and E, and two closely related phyB-type phytochromes, phyB1 and phyB2 (Hauser et al., 1995; Mathews and Sharrock, 1996; Pratt et al., 1995). However, tomato phyB1 and phyB2 are not orthologous to phyB and D in *Arabidopsis*, but represent an independent duplication within the Solanaceae (Pratt et al., 1995). The physiological roles of phyA and phyB in tomato have been characterized by transgenic and mutant analyses (Kendrick et al., 1997; Weller et al., 2000; Weller et al., 2001). The *phyA* mutants of tomato resemble those of *Arabidopsis* (van Tuinen et al., 1995), indicating that phyA has similar functions in both species. Tomato *phyB1* mutants (*tri*), however, are not phenotypically equivalent to either *Arabidopsis phyB* or *phyD* mutants (Tatusov et al., 1997). In particular, in contrast to the *Arabidopsis phyB* mutant, tomato *phyB1* mutants retain a strong response to end-of-day far red light (Kerckhoffs et al., 1997).

Phytochrome A and cry1 have been identified as the major photoreceptors mediating blue light-induced de-etiolation in tomato, and act under low and high irradiances, respectively. Development of *phyA phyB1 phyB2 cry1* quadruple mutants under white light is severely impaired, and seedlings die before flowering. In addition to its effects on de-etiolation, cry1 is

active in older plants, and influences stem elongation, apical dominance, and the chlorophyll content of leaves and fruit (Weller et al., 2001). Less is known about the properties and functions of the other photoreceptors in tomato.

Several light-hypersensitive mutants have been described in tomato. Among these, mutants carrying the monogenic recessive *high pigment* (*hp1* and *hp2*), *atroviolacea (atv)*, *Anthocyanin fruit (Af)* and *dark green* (*dg*) mutations are characterized by their exaggerated light responsiveness. Seedlings of many of these mutants display higher anthocyanin levels and shorter hypocotyls in comparison to their wild-type counterparts (Mochizuki and Kamimura 1984). These homozygous mutants are often also characterized by higher fruit and foliage pigmentation (Wann, 1997). The high fruit pigmentation of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene and flavonoids in the mature red-ripe fruit. The other light hyperresponsive mutants have been only poorly characterized and cloning of these genes may provide more information regarding downstream regulators of photoreceptors. These mutants have therefore been analyzed in more detail in Chapter 2.

1.7 Biotechnological Applications of Photomorphogenesis Research

Research over the last decades has steadily revealed the basic cellular mechanisms that are involved in light signalling (Schafer and Bowler, 2002), and an extraordinarily sophisticated picture has emerged of how plant responses are modulated by incident light (see above). There has also been considerable interest in the use of photomorphogenesis research for biotechnological applications.

The findings described above demonstrate that plant photoreceptors play a key role in modulating plant development for adaptation to specific environmental niches. Consequently, the flexibility in developmental programming that results from the fine-tuning of the phytochromes and cryptochromes must have been an important driving force during angiosperm evolution.

Modern agricultural practices place different constraints on plant growth that have often not been selected for during plant evolution. For example, shade avoiding species grown in dense

monocultures display yield penalties to reproductive organs such as fruits and seeds, due to increased resources being allocated to elongation growth. However, by modifying light perception and signalling, plant responses can be optimized for maximizing agricultural productivity. Several examples where this has been achieved are described below.

1.7.1 Modulation of day length perception

Daylength is an environmental cue associated with seasonal changes, and measurement of photoperiod allows the precise control of the onset of flowering in many plant species. In landmark experiments, Garner and Allard reported in 1920 (Garner and Allard, 1920) that some plants flower faster when the photoperiod is short, that others flower when the photoperiod is long, and that others can flower independently of daylength. These three groups are now known, respectively, as short-day plants, long-day plants, and day-neutral plants.

Significant advances have now been made in understanding how photoreceptors are used by plants to control photoperiod measurement, using principally Arabidopsis as a long-day model and rice as a short-day model (reviewed in Yanovsky and Kay, 2003). What has emerged is the importance of the phyA and cry2 photoreceptors, at least in Arabidopsis.

Following Garner and Allard's discovery that many plants important for agriculture have critical day length requirements for flowering and fruiting, the optimization of photoperiod became a standard practice. For example, new crops were grown only in areas and during seasons with the appropriate length of day, and greenhouse-grown crops were often supplemented with artificial light to extend photoperiod or were covered with black cloth to shorten it. Nowadays, the artificial manipulation of photoperiod is routinely applied to an enormous range of economically important plant species, from crop plants, to flowering plants and trees. Without doubt, it has brought billions of dollars of benefits to farmers, horticulturists, and breeders.

Furthermore, utilization of knowledge derived from the physiology-based experiments of phytochrome action performed largely in the first half of the 20th century have had dramatic impacts on the flower industry. For example, pulsed light is often used to substitute constant

light, and a single pulse of light during the night can be used to inhibit flowering in some species. These practices exploit some of the now well known characteristics of the phytochromes; in the above two examples that phytochrome responses have an escape time and that phytochrome concentrations build up during the night but can be reduced significantly by a single light pulse. Again, such practices permit enormous cost savings to growers.

The molecular mechanisms controlling phytochrome behaviour are now understood to some extent. In principle, it has therefore become feasible to modify day length perception by the judicious manipulation of key regulators. This allows a high level control of plant growth and could optimize it to the particular conditions available. Many examples of manipulation of key photoperiod regulators have been reported (e.g., manipulation of CONSTANS levels is particularly dramatic (Yano et al., 2000; Liu et al., 2001)) nothing has yet been reported in a biotechnological context, in spite of its feasibility. However overexpression of AtCO in potato resulted in a delayed tuberization phenotype, suggestive of a function for CONSTANS in the photoperiodic pathways controlling tuber formation (Martinez-Garcia et al., 2002).

1.7.2 Modulation of shade avoidance responses

Competition for light energy is a very important factor controlling plant architecture. As described above, when plants are grown in close proximity the shade avoidance response is activated, manifested by a dramatic increase in extension growth at the expense of leaf growth, storage organ production, and development of reproductive organs. These architectural changes are a serious problem for agriculture, because they reduce the amount of useful material that can be harvested. Concomitantly, the production of large quantities of unwanted material in a crop carries a cost in terms of wasted nutrients, which must consequently be supplied at high levels. Furthermore, it has been found that competition from weeds is directly attributable to the induction of shade avoidance responses in crop plants rather than to competition from resources (Smith, 1994).

Studies in phytochrome-deficient mutants have demonstrated convincingly that shade avoidance responses are mediated by light-stable Type II phytochromes, in particular by phyB in Arabidopsis (Quail et al., 1995). This is primarily because the far red-enriched light

environments in shaded conditions causes a reduction in PfrB:PrB ratios, which results in increased stem elongation and other responses associated with shade avoidance.

Harry Smith and colleagues have utilized this information to generate transgenic plants in which the shade avoidance response has effectively been disabled. This was achieved in transgenic tobacco plants overexpressing a *PHYA* cDNA from oat (Robson et al., 1996). In contrast to the endogenous phyA, the transgene-derived oat phyA remained at high levels, resulting in considerably higher levels of phyA in extracts from light-grown transgenic plants compared to wild-type plants (Smith, 1994; Robson et al., 1996). Presumably, the processes regulating the light-dependent degradation of endogenous phyA were not being utilized to control levels of the exogenous phyA.

Phenotypically, transgenic oat *PHYA* overexpressing plants did not show the increased stem elongation in far red-enriched white light (McCormac et al., 1992a,b), one of the characteristic features of the shade avoidance response. This remarkable result suggested that phyA must normally be removed in mature plants, in order that the Type II phytochromes (principally phyB) can activate the shade avoidance response. Furthermore, it suggested a means whereby the shade avoidance response could be effectively inactivated.

Field trials of these tobacco plants grown at different densities indeed demonstrated that the densely grown plants did not display the typical shade avoidance responses of wild-type plants. Furthermore, the plants elongated even more slowly in dense plantings compared with sparse plantings (Robson et al., 1996), which could also be attributed to the substitution of the normal phyB responses by the exogenous phyA. These changes in light perception resulted in significant increases in the harvest index of leaves from the transgenic plants compared to the wild-type plants.

The results described above demonstrate the overriding importance of phytochrome-mediated shade avoidance compared with other potential factors associated with plant performance at high densities, at least in the monoculturing situation that typifies current agricultural practices. The transgenic suppression of shade avoidance opens up possibilities for the conditional modification of architecture based upon a plant's intrinsic ability to measure plant density. This can potentially lead to improvements in crop yield, and could be used to control

density-dependent dwarfing, which could even reduce problems associated with lodging and nutrient utilization.

1.7.3 Modulation of fruit ripening

The influence of light on the development of climacteric fruits such as tomato is well known, and in the 1950s an important role for Type II phytochromes was demonstrated in the process (Piringer and Heinze, 1954). Subsequently, the importance of individual photoreceptors was revealed by the identification and study of photoreceptor mutants in single, double, and triple combinations (Weller et al., 2000). For example, the loss of phyB2 in a *phyAphyB1* background results in a striking reduction in chlorophyll content of immature fruits as well as a marked increase in truss length as a result of increased distance between fruits on the inflorescence axis. The loss of cry1 in this same background also results in a reduction of chlorophyll in immature fruits but has no effect on truss architecture.

One could therefore speculate that an increase in photoreceptor activity in fruits would lead to enhanced pigmentation. This is of particular commercial interest because tomato fruit and derived products (e.g., ketchups, juices, soups, and sauces) are important sources of vitamins and carotenoids and are consumed by millions of people each day. Consequently, tomato cultivation and processing are billion dollar industries both in the USA and in Europe. The major carotenoid pigment found in ripe tomato fruits is lycopene, which is a potential cancer chemopreventative, particularly for prostate cancer (Kucuk et al., 2002; Miller et al., 2002). Lycopene is also a potent scavenger of oxygen radicals. In addition, beta-carotene, which is derived from lycopene, is a pro-vitamin A and its deficiency is estimated to cause the deaths of between 1 and 2 million children each year (Humphrey et al., 1992).

A clear demonstration that the manipulation of light signalling pathways can enhance tomato fruit carotenoid levels is represented by the tomato *high pigment* (*hp*) mutants, which are characterized by dark green immature fruits that develop into mature fruits containing more than twice the normal levels of lycopene and β-carotene (Mustilli et al., 1999). Mutations in the tomato homologue of the *DET1* gene were found to be responsible for one of these mutant loci, *hp2* (Mustilli et al., 1999). Previous studies in Arabidopsis had indicated that DET1 was an important negative regulator of light responses (Chory et al., 1989; Pepper et al., 1994).

In spite of the enhanced nutritional quality of *hp* mutant fruits, they have never been commercialized because of the collateral negative effects that the mutation has on the vegetative parts of the plants, resulting in plants with bushier growth habits and reduced yields. This is likely to be a general problem when attempting to manipulate such general processes as light signalling.

Indeed, overexpression of oat phytochrome in tomato increased pigmentation in fruits, but also increased dwarfness (Boylan et al., 1989). It is therefore apparent that more sophisticated strategies are required to utilize the knowledge acquired from photomorphogenesis research to manipulate tomato fruit ripening. An example of such an advanced technology is described in Chapter 4.

1.8 Aims

In Chapter 2, several previously described light hyperresponsive mutants of tomato, namely *atroviolacea (atv)*, *Anthocyanin fruit (Af)*, *dark green* (*dg*) and *Punctate* (*Pn*), were selected to study their responses to light at the physiological and molecular levels. The well-characterized *high pigment (hp1* and *hp2)* mutants were utilized for comparison with these other largely understudied mutants. The objective of this study was to reveal the general characteristics of these mutants in response to white light and broadband light of different wavelengths, in order to reveal whether they are defective in responses mediated by specific photoreceptors or whether they show more general defects. It was hoped that this study would reveal which mutants were the most interesting for future studies and that it would provide clues as to the nature of the mutated genes by analogy with what is known about light signalling from other better characterized photomorphogenesis mutants in tomato and Arabidopsis.

Chapter 3 focuses on a reverse genetics approach to study the function of DET1 in tomato. DET1 is a negative regulator of light signalling and has been cloned previously from both *Arabidopsis* and tomato, although its function in light signal transduction remains unclear. In an attempt to better characterize the function of this protein during plant development, different forms of the tomato *DET1* gene were utilized to generate transgenic tomato plants, which were subsequently analysed using molecular and biochemical approaches.

Chapter 4 is a continuation of the reverse genetics methods utilized in Chapter 3, and the experiments were aimed at inhibiting endogenous *DET1* gene expression specifically within the fruits by post-transcriptional gene silencing, in order to eliminate the negative effects on plant development caused by loss of DET1 activity but to maintain the high carotenoid phenotypes of *hp2* mutant fruits, which may be of significant biotechnological interest due to the enhanced nutritional value of such fruits.

CHAPTER 2

Physiological and Molecular Characterization of Light Hyperresponsive Tomato Mutants

2.1 Summary

In this Chapter, several light-hyperresponsive mutants of tomato (*Solanum lycopersicon*) have been selected that were described long ago but have never been characterized in detail. These are denoted *atroviolacea* (*atv*), *Anthocyanin fruit* (*Af*), *Punctate* (*Pn*) and *dark green* (*dg*). A physiological characterization of these mutants has been done in natural white light and in broadband light of different wavelengths (blue, red and far/red) using the well characterized *high pigment* (*hp*) mutants for comparison. *atv* mutants showed exaggerated light hyperresponsiveness in natural white light in terms of anthocyanin content in stems and leaves, similarly to *hp* mutants, although no obvious phenotypes were observed in fruits. Like the *hp* mutants, *atv* mutants developed plastids in the roots of light grown seedlings, and partial plastid development was observed in cotyledons from seedlings grown in total darkness. These results were confirmed by both confocal and electron microscopy. In contrast, both *Af* and *dg* showed more specific phenotypes in the fruits but less so in the vegetative parts of the plant. In the case of *Af,* higher anthocyanin contents were observed in the sub-epidermal layers of the fruits whereas *dg* mutants developed fruits with dark green pigmentation in their shoulders. Both *atv* and *Af* seedlings showed slightly higher anthocyanin and chlorophyll contents than their respective wild-type seedlings in natural white light and broadband experiments but no differences were observed in hypocotyl length. The mutants *dg* and *Pn* did not differ much from respective wild-type seedlings in any light condition. In *atv* mutant seedlings, both *CAB* and *CHS1* gene expression were similar in the dark and in white light-grown seedlings compared to wild-type seedlings. In the *Af* mutant, *CAB* gene expression was similar to wild-type when grown in the dark whereas *CHS1* gene expression was slightly up-regulated in white light-grown seedlings. Cloning of these genes may therefore identify new signalling intermediates and may provide further insights into the function of photoreceptors during fruit development.

2.2 Introduction

The use of tomato mutants in photomorphogenesis research is relatively new, but has expanded rapidly. Many new mutants are now available, and in a many cases the molecular nature of the genetic lesions are known. Reviews describing the various mutants in higher plants are those of Nagy and Schafer (2002) and Hardtke and Deng (2000). A great deal of knowledge has been gained regarding light signal transduction in plants from studies

involving the characterization of mutations affecting photomorphogenesis in *Arabidopsis* and tomato. In *Arabidopsis*, where most work has been done, these mutants generally fall into two classes, those showing light insensitive phenotypes and those displaying constitutive light responses.

Photomorphogenic mutants would be expected to have strong pleiotropic phenotypes, because many responses are regulated by light and are expected to be modified when the mutation affects the photoreceptor itself or steps immediately following the perception of light. The similarity of phenotypes of different light-insensitive mutants in white light led to the identification of photoreceptors in *Arabidopsis* (Koornneef *et al.*, 1980). Many phytochrome and cryptochrome genes were subseqently cloned and characterized from studies involving light insensitive mutants.

Similarly, many signal transduction intermediates were identified by studying light-responsive mutants. Chory *et al.* (1989) isolated mutants that, when grown in complete darkness, had a number of properties characteristic of light-grown plants, such as short hypocotyls, an open apical hook, the development of primary leaves, etc. Mutants with this phenotype in *Arabidopsis* have been called *de-etiolated* (*det*) and *constitutively photomorphogenic* (*cop*) (Deng *et al.*, 1991; Deng and Quail, 1992). Other mutants have been shown to be more responsive to light than corresponding wild-type plants. Because these mutants have a normal etiolated phenotype in darkness they represent a class different from the constitutive group described above. Examples of this class are the tomato *high pigment* mutants represented by two loci, *hp1* (Peters *et al.*, 1989) and *hp2* (Peters *et al.*, 1992).

Furthermore several other mutants of tomato have been identified which, among other phenotypes, display important morphological differences in the fruits. Tomato is also useful for studying the light-regulated control of anthocyanin biosynthesis because in tomato anthocyanin production is strictly light dependent (Von Wettstein-Knowles, 1968). We have selected some of the tomato mutants on the basis of their exaggerated phenotypes when grown in the light. In tomato no constitutive mutants have ever been found. However, mutants with an altered or an exaggerated light response have been identified, such as *high pigment (hp1* and *hp2)*, *atroviolacea (atv)* and *Anthocyanin fruit (Af)*. Cloning of the *HP1* and *HP2* gene revealed that they encode the tomato homologue of *Arabidopsis* DDB1 and DET1

respectively (Liu et al., 2004; Mustilli et al., 1999). The *hp2* mutation causes substantially different phenotypes compared to *Arabidopsis*. Characterization of other light-hypersensitive mutants in tomato may identify new components of signal transduction pathways specific for phytochrome or the other photoreceptors or may reveal differences in the regulation of light signalling between *Arabidopsis* and tomato.

2.3 Description of the mutants

2.3.1 high pigment (hp)

The monogenic recessive *high pigment* mutants of tomato are characterized by short hypocotyls and intense anthocyanin pigmentation in seedlings (Figure 2.1). They develop dark green immature fruits (Figure 2.1d) and mature fruits have higher lycopene and carotenoid contents than wild-type fruits (Mustilli *et al.*, 1999). Recently it was shown that the *hp* mutants are also able to develop anthocyanin in the roots. The *hp1* mutant was originally discovered as a spontaneous mutant in 1917 at the Campbell Soup Company farms (Riverton, N.J.) (Reynard, 1956) and the $hp1^{w}$ mutant appeared among the progeny of a plant raised from ethyl methanesulfonate (EMS)-treated seeds of the genotype GT (Peters et al., 1989). The *hp2* mutant was reported in the Italian San Marzano variety in 1975 (Soressi, 1975). Additional alleles of both were described thereafter (Peters et al., 1989; Van Tuinen et al., 1997). Some of these alleles, like $hp1^{w}$and $hp2^{j}$ display a stronger phenotype than earlier described alleles. The $hp2^{j}$ mutant was found among progeny of a T-DNA-transformed plant (cv. Money Maker) (van Tuinen et al., 1997). Despite some initial confusion, it is now clear that there are two *HP* genes *HP1* and *HP2* in the tomato genome, that map to chromosomes 2 and 1, respectively (van Tuinen et al., 1997; Yen et al., 1997). At each of these loci, two of the above mentioned mutant alleles have been identified: *hp1* and $hp1^{w}$, *hp2* and $hp2^{j}$ (Kerckhoffs et al., 1997[a]; van Tuinen et al., 1997).

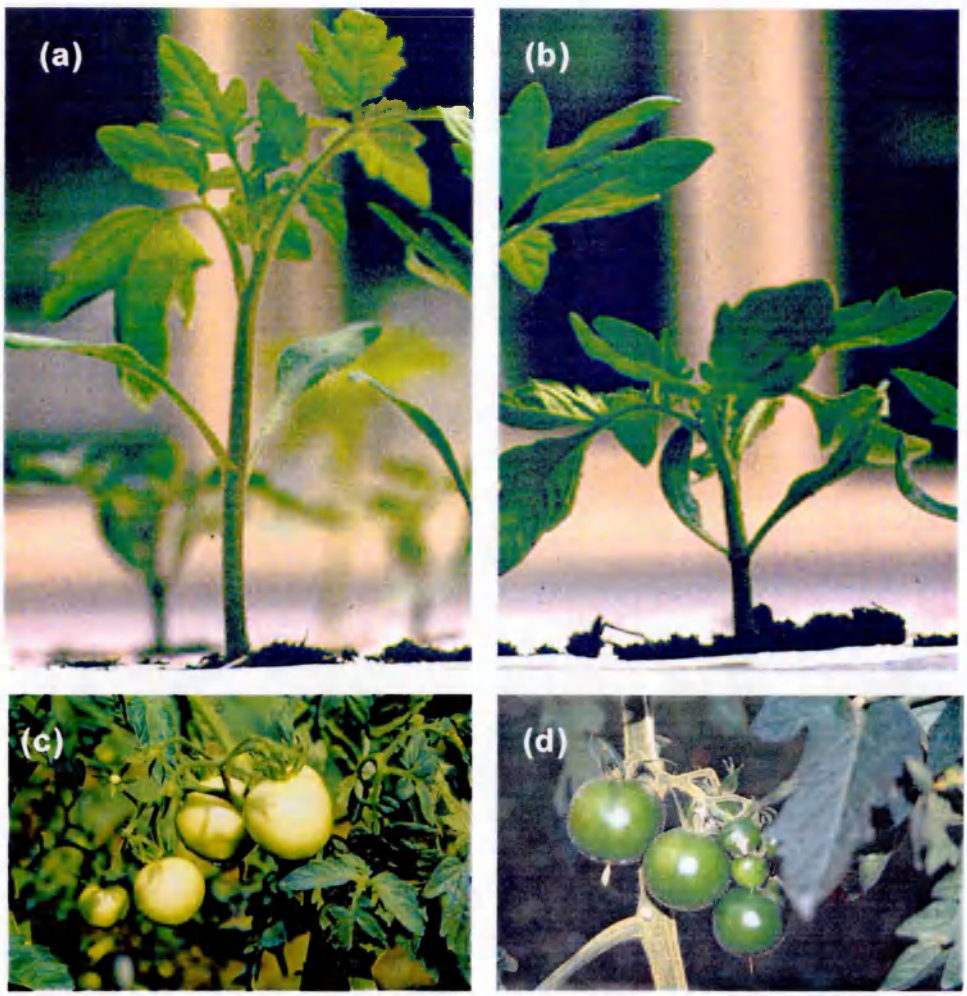


Figure 2.1. Phenotypes of *high pigment2* (*hp2*) mutants grown in natural white light conditions. Seedling phenotype of Money Maker (a) and *hp2* mutants (b). Immature fruits of Money Maker (c) and *hp2* mutants (d).

Molecular genetic analysis of *hp2* mutants showed that the *HP2* gene encodes the tomato homologue of the nuclear protein DET1 from *Arabidopsis*, and is therefore denoted TDET1 (Mustilli et al., 1999). The *hp2* mutants are phenotypically different from *det1* mutants. All *hp* mutants display an almost normal dark phenotype whereas *det1* mutants show reduced hypocotyl length, opened apical hooks, and enlarged cotyledons when grown in the dark. However, plastid development in darkness in the cotyledons of *hp2* and *hp2^j* seedlings is comparable to that observed in *Arabidopsis det1* mutants (Mustilli et al., 1999). Recent studies have revealed that *HP1* encodes the tomato homologue of the gene encoding UV-damaged DNA Binding protein 1 (DDB1) (Liu et al., 2004), which has been shown to physically interact with DET1 in *Arabidopsis* (Schroeder et al., 2002).

2.3.2 atroviolacea (atv)

The *atv* mutant derives from a segregant in a natural population of *Lycopersicon pimpinellifolium* from the Galapagos (Rick, 1961) but is almost certainly a *L. cheesmanii* accession (Kerckhoffs et al., 1997). Thereafter, *atv* was backcrossed twice with wild type Ailsa Craig. *atv* is characterized by high levels of anthocyanin in stems and leaves (Figure 2.2). In red light, *atv* mutants were reported to exhibit twice as much anthocyanin as in blue light, which suggests that the *atv* mutation may affect specifically phytochrome responses (Kerckhoffs et al., 1997). *atv* mutant fruits are comparable to wild-type fruits (Figure 2.2c). Genetic analysis has shown that *atv* maps to chromosome 7 (Rick *et al.*, 1968).

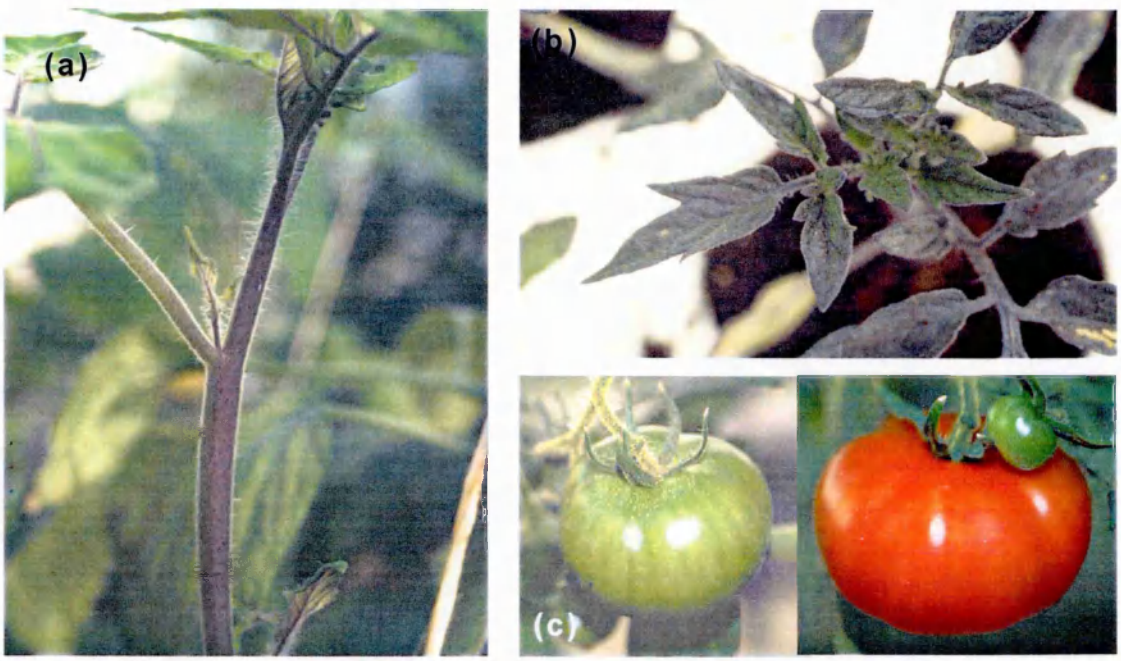


Figure 2.2. Phenotypes of *atroviolacea* (*atv*) mutants grown in natural white light conditions. (a) Anthocyanin in the stem of *atv* mutant plants. (b) Anthocyanin in the leaves of *atv* mutant plants. (c) Immature and mature fruits of *atv* mutant plants, which resemble wild-type Ailsa Craig fruits.

2.3.3 Punctate (Pn)

The dominant mutant *Pn* was first described by Rick in 1966. In the mutant *Pn*, anthocyanin is frequently observed in the cortical cells beneath the hairs, the basal cells and the stalk cells of the hairs. The large leaf hairs filled with anthocyanin give the leaf its punctate appearance. In wild-type plants anthocyanin is limited to the cortical cells beneath the hairs (von Wettstein-Knowles, 1968). The gene is located on chromosome 8 (Rick, 1966) and is allelic with the anthocyanin-deficient mutant *al* (Butler, 1973).

2.3.4 Anthocyanin fruit (Af)

This mutant develops very high levels of anthocyanin in the fruit indicating that this mutation might affect only the fruits (Figure 2.3). Phenotypically, the *Af* mutant is influenced by light and temperature conditions.

The strongest expression of the character is observed under intensive light and low temperature. Fruits or parts of fruits which are in the shade do not display anthocyanin pigmentation (Georgiev, 1972). From genetic analysis it is known that this is a dominant mutation but the map position is unknown. *Af* was originally selected in segregating generations from a cross between *Solanum lycopersicon* and *L. chilense* (Georgiev, 1972). The wild-type background is unknown and so for this reason a backcross with Money Maker (*Af/MM*) has been generated and studied here.

2.3.5 dark green (dg)

The *dg* mutant was first described in trellised plantings of the 'Manapal' tomato (*Lycopersicon esculentum*) by Konsler in 1973. A *dg* mutant with background Walter was registered in 1991, although it was unclear whether this is the same mutation as *dg* Manapal. For a long time it was thought that *dg* was allelic with the *hp* mutants, and recently it was shown that *dg* 'Manapal' and *hp1* are separate loci (Jones et al., 2001), but is allelic to *hp2* (Levin et al., 2003). The *dg* 'Walter' mutant has not been described genetically. *dg* fruits have dark green shoulders compared to wild-type fruits, but from the bottom the fruits appear wild type in appearance (Figure 2.4).

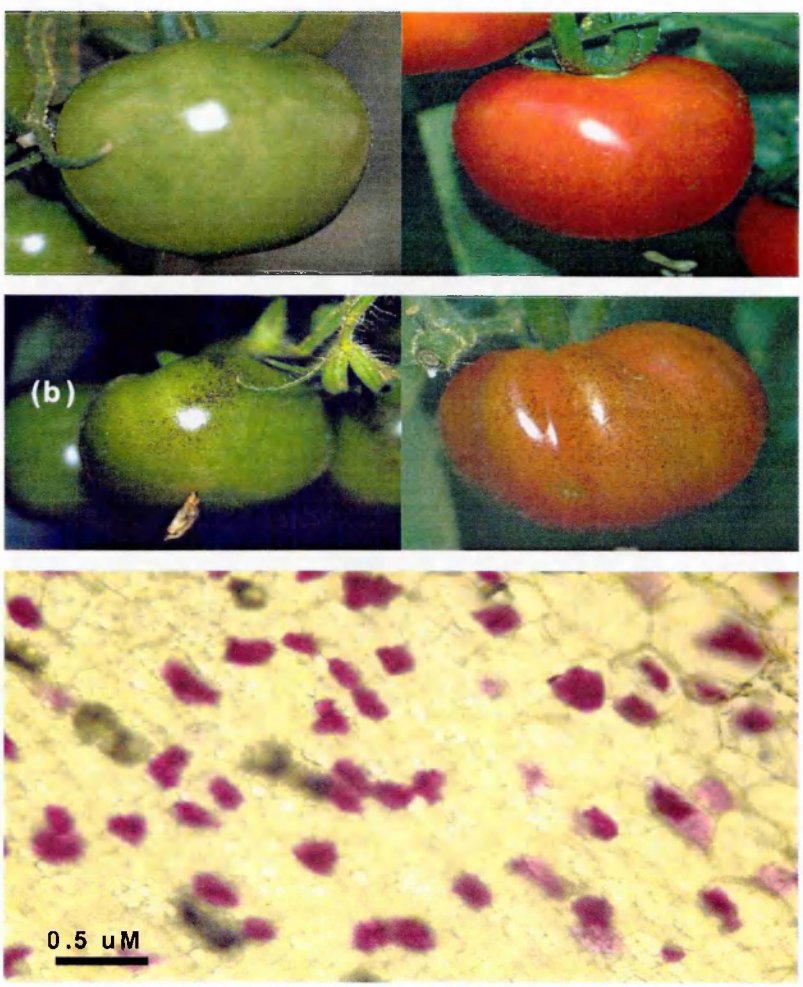


Figure 2. 3. Phenotypes of *Anthocyanin fruit* (*Af*) mutants grown in natural white light conditions. (a) Immature and mature fruits of wild-type Ailsa Craig. (b) Immature and mature fruits of *Af* mutants. (c) Cross section of *Af* fruit and distribution of anthocyanin in sub-epidermal cells. Scale bar = 10mm

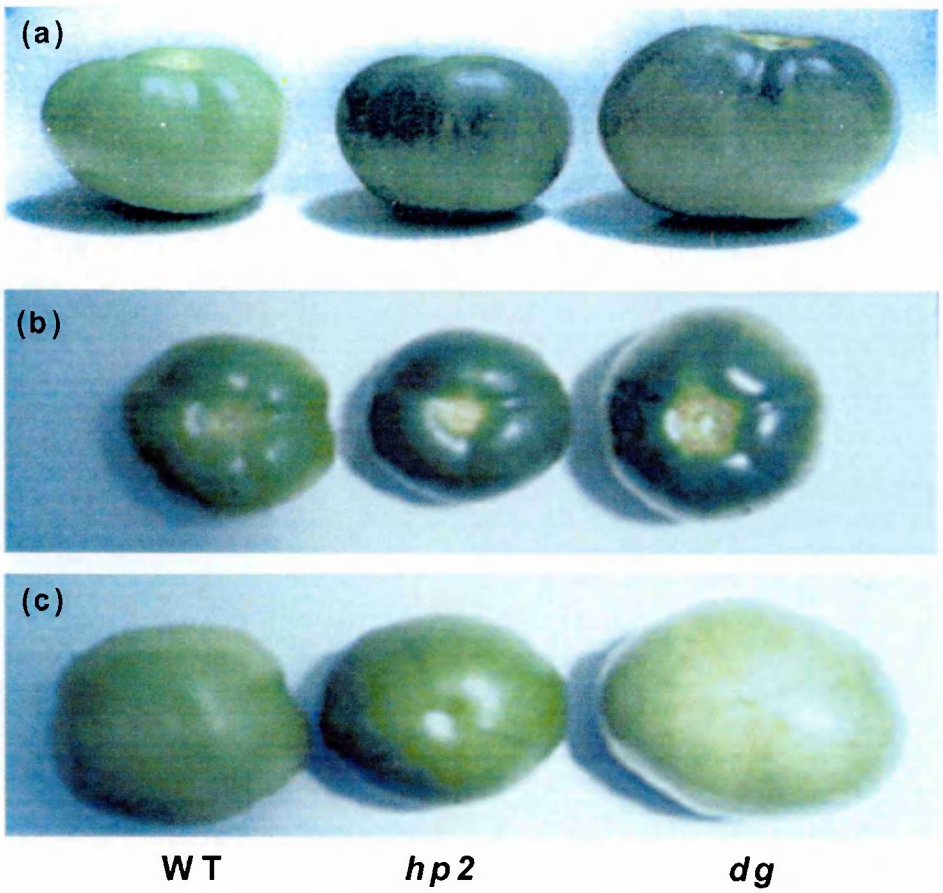


Figure 2. 4. Phenotypes of *dark green* (*dg*) mutants. (a) Side view of immature fruits of wild type (WT), *hp2*, and *dg*. (b) Top view of immature fruits of wild type (WT), *hp2*, and *dg*. (c) Bottom view of immature fruits of wild type (WT), *hp2*, and *dg*. The *dg* fruit resembles *hp2* fruits when seen from the side or top and resembles wild type when seen from bottom. The dark green phenotype only appears in the shoulder region of the fruits.

The aim of this work was to perform a better characterization of the above-mentioned mutants compared to their wild types. This has been done physiologically by measuring hypocotyl length and plant height and by determining chlorophyll and anthocyanin content at different stages of plant development and in different light conditions (white, red, blue, far-red light and dark). Cytological characterization has also been made to determine the location of anthocyanin and chloroplasts in the plants using light microscopy and laser scanning confocal microscopy. Furthermore, the ultra-structure of the chloroplasts has been examined by transmission electron microscopy, and light-regulated gene expression has been studied in seedlings grown in white light.

2.4 Results

2.4.1 Artificial White Light Experiments

As described in Materials and Methods, seedlings were grown on agar medium in magenta boxes and analyzed after 14 days growth in a 16-hour photoperiod. These artificial white light experiments were repeated three times. Although the measured quantities were variable, the trends from each experiment were generally the same. Therefore only the results from artificial white light experiment number two are presented here. Hypocotyl length was measured, and anthocyanin content of hypocotyls and cotyledons was determined. Chlorophyll content of roots and cotyledons was also determined. Hand-cut sections were made to examine anthocyanin distribution by light microscopy, and laser scanning confocal microscopy for the presence of chloroplasts in roots. Finally, roots and cotyledons were prepared for electron microscopy to visualize plastid ultrastructure. This work was performed with the help of Ageeth van Tuinen and Christy Efde.

2.4.1.1 Hypocotyl Length

As shown in Figure 2.5 the *hp* mutants were clearly shorter than their wild-type counterparts. The hypocotyl length $hp1^{w}$ mutant seedlings was almost half that of its wild-type GT and hypocotyl length of $hp2^{j}$ mutant seedlings was less than one third the hypocotyl length of corresponding wild-type seedlings. The differences between the other mutants and their wild

types are less apparent. The original *Af* mutant seedlings were shorter than MM seedlings, but the hypocotyl length of *Af*/MM seedlings was intermediate between *Af* and MM seedlings. The hypocotyl length of *dg* mutant seedlings was always a little shorter than its wild-type WA seedlings. In the different experiments, the hypocotyl length of the *Pn* mutant seedlings was not reproducibly different compared to the hypocotyl length of wild-type AC seedlings. Although there were differences in hypocotyl length, the average values were in the same range and there was no clear trend in the different experiments. It therefore appears that the hypocotyl length of *Pn* mutant seedlings was the same as wild-type seedlings. On the contrary, although the hypocotyl length of the *atv* mutant seedlings varied from experiment to experiment, the plants were generally shorter. However, this was not statistically significant.

2.4.1.2 Anthocyanin Content

Anthocyanin content in the hypocotyls of $hp1^{w}$ mutant seedlings was 4-times higher than in GT seedlings whereas $hp2^{j}$ mutant seedlings had anthocyanin contents almost 8-times higher than that of MM seedlings (Figure 2.6a). In *Af*/MM seedlings hypocotyl anthocyanin content was 2.5 times higher than in MM seedlings. The original *Af* mutant seedlings have anthocyanin content similar to the hypocotyls of *Af*/MM seedlings, but are generally more variable. The anthocyanin content of *atv* seedling hypocotyls was 4.5-5 times higher than AC seedlings, similar to *hp* mutant seedlings (Figure 2.6a). The other mutants did not differ much from wild-type seedlings. The anthocyanin content of the cotyledons was always much lower than in the hypocotyls. However, anthocyanin levels within cotyledons of each of the mutant seedlings compared to wild-type seedlings showed essentially the same pattern as with the anthocyanin content of the hypocotyls (Figure 2.6b), e.g., $hp1^{w}$ mutant seedlings have a 4.5-times higher level of anthocyanin than GT seedlings and $hp2^{j}$ mutant seedlings have up to 9-times more anthocyanin in the cotyledons compared to MM seedlings. The original *Af* mutant seedlings have slightly more anthocyanin in the cotyledons than do MM seedlings. However, *Af*/MM mutant seedlings have a little more anthocyanin in the cotyledons than MM seedlings. Anthocyanin content in the cotyledons of *dg* mutant seedlings was the same as wild-type WA seedlings. *Pn* mutant seedlings have a little more anthocyanin than AC seedlings, while in *al* mutant seedlings anthocyanin content was half of that found in AC seedlings. *atv* mutant seedlings have more anthocyanin than AC seedlings in the cotyledons, and the difference is more significant than for *Af* and *Pn* mutant seedlings (Figure 2.6b).

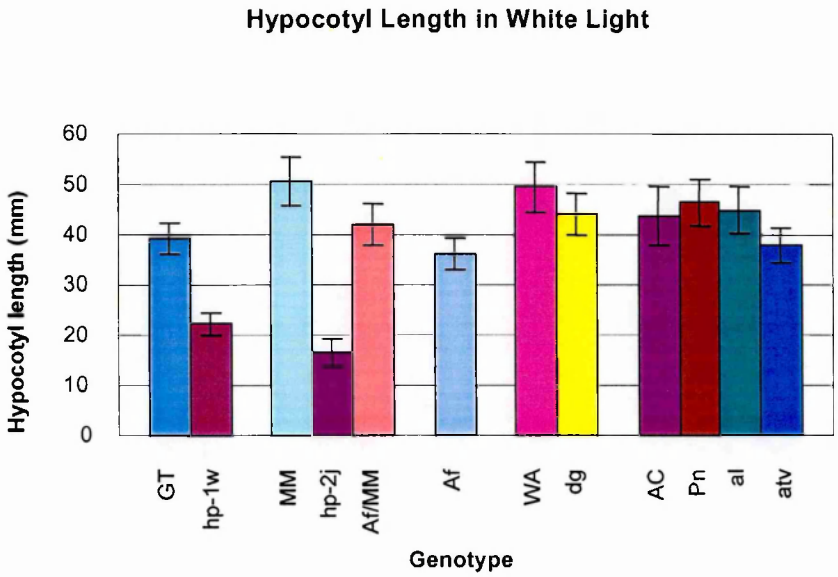
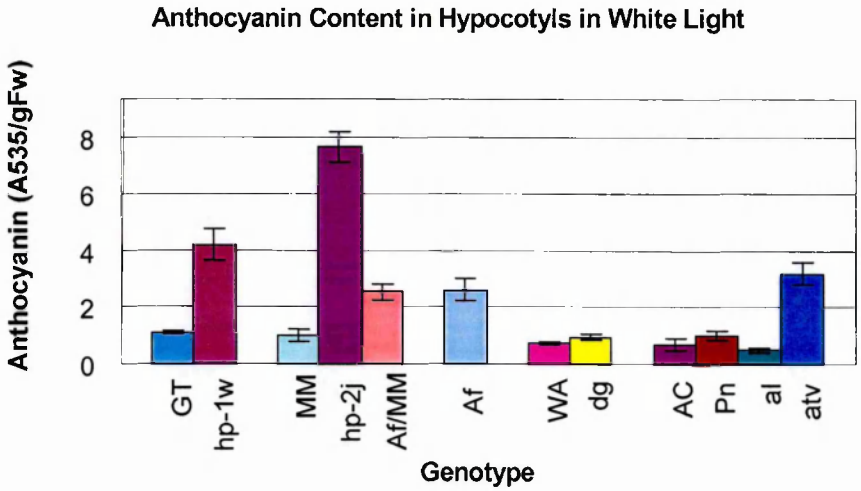


Figure 2.5. Hypocotyl length of different mutants and their respective wild-type seedlings. Hypocotyl lengths of seedlings grown in artificial white light at 25 ^{0}C for 14 days in a 16-hr-light and 8-hr-dark photoperiod. Values are the means of 10 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

(a)



(b)

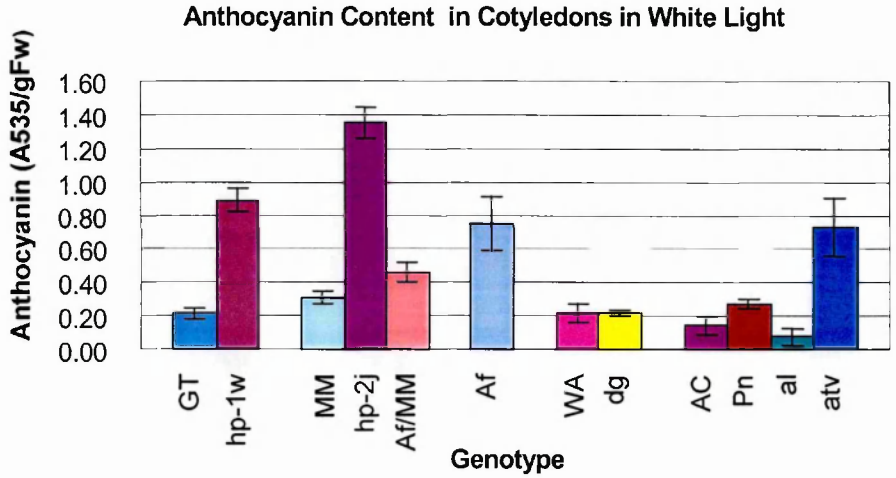


Figure 2.6. Anthocyanin content from hypocotyls and cotyledons of different mutants and their respective wild-type seedlings. (a) Anthocyanin content of hypocotyls of seedlings grown in artificial white light at 25 ^{0}C for 14 days in a 16-hr-light and 8-hr-dark photoperiod (b) Anthocyanin content of cotyledons of seedlings grown in artificial white light at 25 ^{0}C for 14 days in a 16-hr-light and 8-hr-dark photoperiod. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated three times. Error bars indicate ±SE.

2.4.1.3 Chlorophyll Content

For most mutant seedlings the chlorophyll content of cotyledons did not differ from wild-type seedlings. *hp1*^{w} mutant seedlings displayed more chlorophyll in experiments 1 and 3 than wild-type seedlings, although not in experiment 2, whereas *hp2*^{j} mutant seedlings consistently had slightly more chlorophyll than wild-type MM seedlings (Figure 2.7). After two weeks growth in magenta boxes in white light the roots of *hp1*^{w}, *hp2*^{j} and *atv* mutant seedlings became visibly green. The roots contained high levels of chlorophyll whereas roots from wild-type seedlings have no chlorophyll at all (Figure 2.8). None of the other mutant seedlings developed chlorophyll in the roots under these conditions.

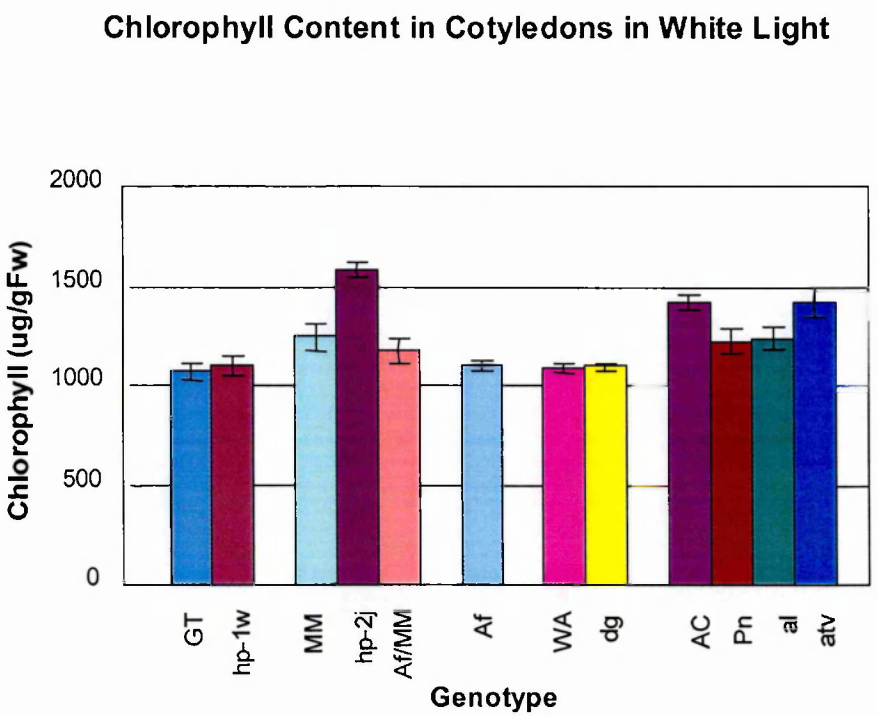


Figure 2.7. Chlorophyll content from cotyledons of different mutants and their respective wild type seedlings. Chlorophyll content of cotyledons was measured in seedlings grown in artificial white light at 25 ^{0}C for 14 days in a 16-hr-light and 8-hr-dark photoperiod. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE

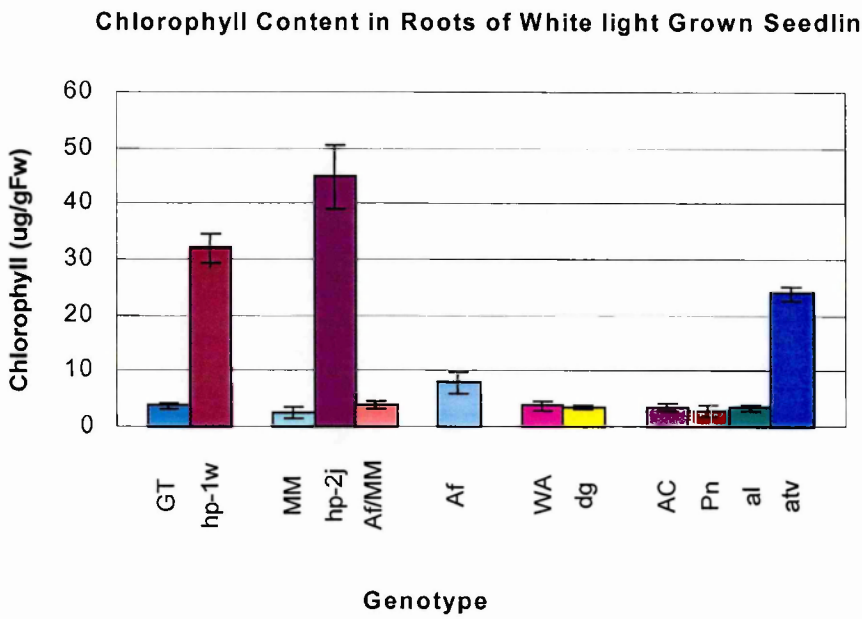


Figure 2.8. Chlorophyll content in roots of different mutants and their respective wild type seedlings. Chlorophyll content of roots in seedlings grown in artificial white light at 25 ^{0}C for 14 days in a 16-hr-light and 8-hr-dark photoperiod. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated three times for artificial white light and two times for natural white light. Two roots from each seedling were taken for chlorophyll extraction. Error bars indicate ±SE.

2.4.2 Natural white light experiments

The natural white light experiment (see Materials and Methods) was repeated two times. Tomato seedlings were grown under natural light conditions in a greenhouse near Naples for one month. Hypocotyl length and plant height were measured. Anthocyanin content of hypocotyls and young leaves, and chlorophyll content of young leaves were also measured. Hand-cut sections were made to examine anthocyanin distribution by light microscopy. The results are described below.

2.4.2.1 Hypocotyl Length and Plant Height

The *hp* mutant seedlings were again much shorter than wild-type seedlings (Figure 2.9). Hypocotyl length of $hp1^{w}$ seedlings was shorter than GT seedlings, but the difference in plant height was much more apparent. The hypocotyl length of $hp2^{j}$ mutant seedlings was also clearly shorter than wild-type seedlings and plant height was only half that of the corresponding wild-type plant height. The other mutant seedlings showed less distinctive differences in hypocotyl length and plant height. *atv* mutant seedlings did not differ with wild-type seedlings for either parameter, considering the fact that the *atv* mutant seedlings have more anthocyanin content.

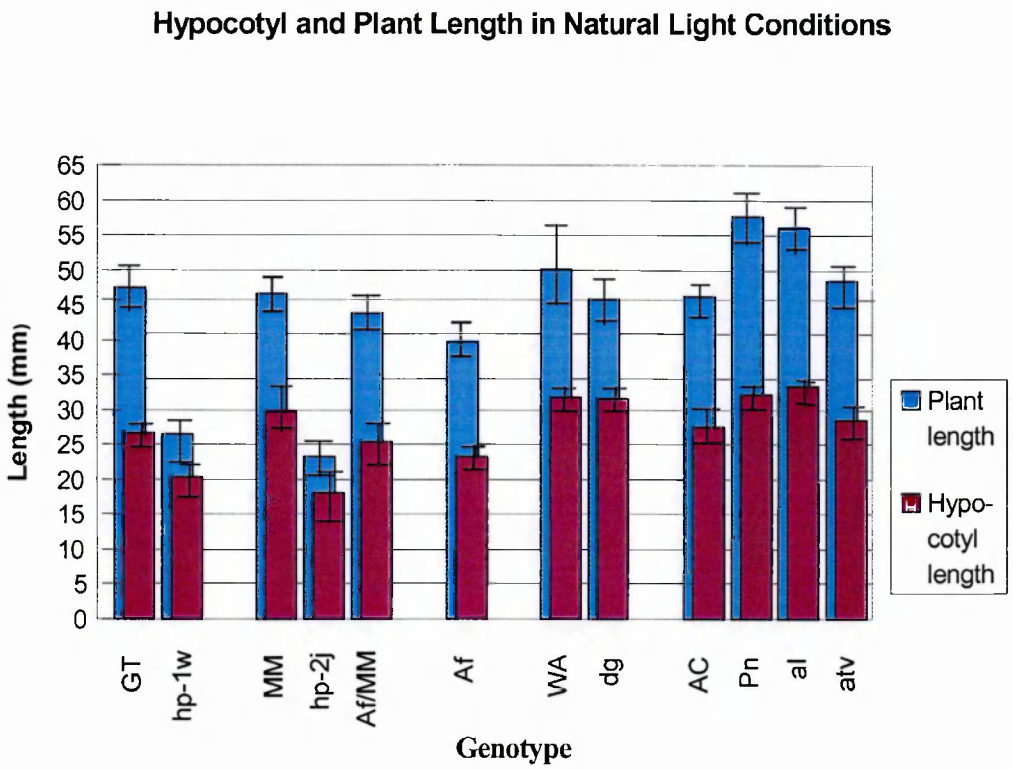


Figure 2.9. Hypocotyl length of different mutants and their respective wild-type seedlings. Hypocotyl and plant length of seedlings grown in natural white light for one month. Values are the means of 10 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

2.4.2.1 Anthocyanin Content

Anthocyanin accumulation in the hypocotyls of *hp1*^{w} mutant seedlings was up to 5-times higher than normal wild-type levels (Figure 2.10). In *hp2*^{j} mutant seedling hypocotyls, anthocyanin content was 2.5-times higher than in MM seedlings. *Af*/MM mutant seedlings showed 1.6-times elevated levels compared with MM seedlings. *Af* mutant seedling hypocotyls had similar anthocyanin content as *Af*/MM seedlings. In the *dg* mutant seedlings, anthocyanin levels were unaltered compared with WA seedlings, whereas in *Pn* mutant seedlings, anthocyanin content was slightly lower than in wild-type seedlings, and in hypocotyls of *al* mutant seedlings, levels were even lower. The *atv* mutant seedlings had clearly elevated levels of anthocyanin, approximately two-times the levels found in wild-type seedlings.

The anthocyanin content of young leaves was always lower than the anthocyanin content in the hypocotyls. Also the differences in anthocyanin content between mutant and wild-type seedlings were smaller (Figure 2.11). *hp1*^{w} seedlings showed the biggest differences with wild-type seedlings, but had only 1.7-times higher anthocyanin levels. In *hp2*^{j} seedlings the anthocyanin content was only slightly higher than in wild-type seedlings. Anthocyanin levels in *Af*/MM seedling leaves were lower than MM seedlings, although the original *Af* mutant seedlings displayed a slightly elevated level of anthocyanin compared with *Af*/MM seedlings. *dg* seedlings again did not show any difference compared with the wild-type seedlings. Anthocyanin content of *Pn* seedlings was a little higher compared with AC seedlings, whereas the *al* mutant seedlings showed negligible levels. Anthocyanin content of *atv* seedling leaves was two-times the amount found in wild type AC seedlings.

2.3.2.3 Chlorophyll Content

No clear differences between mutant and wild-type seedlings were observed in chlorophyll content in young leaves (Figure 2.12).

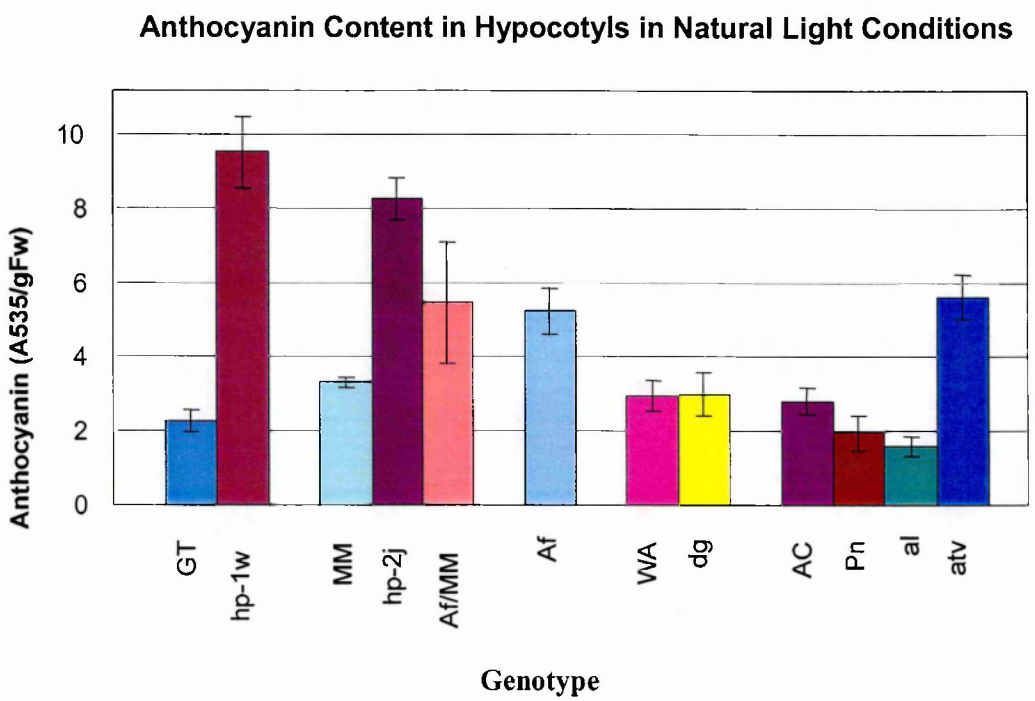


Figure 2.10. Anthocyanin content of hypocotyls of seedlings grown in natural white light for one month. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

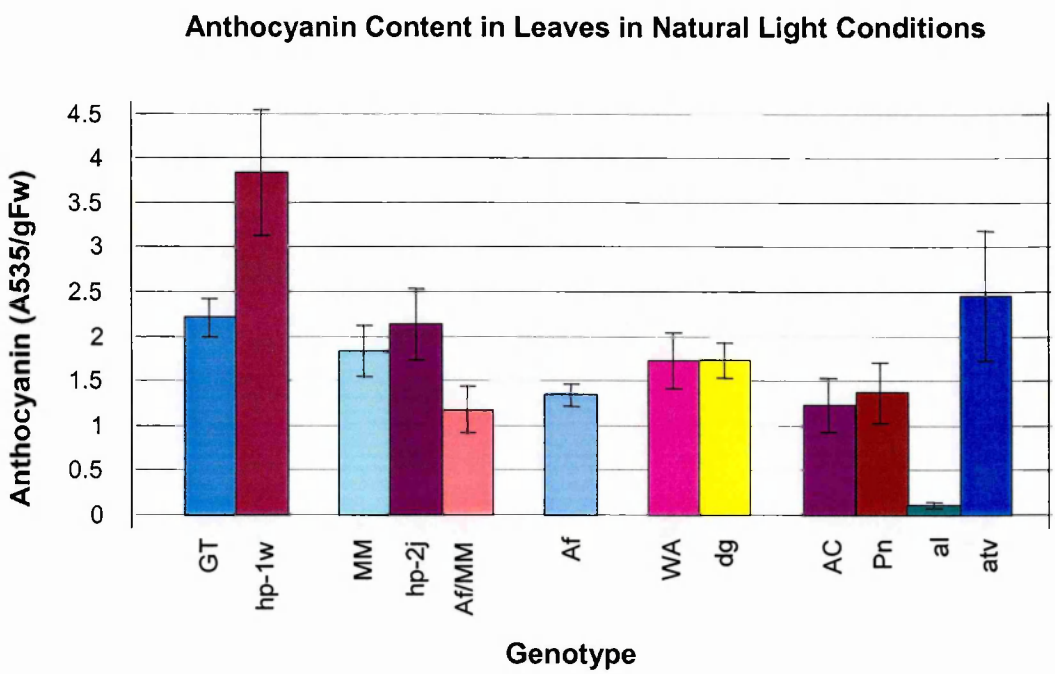


Figure 2.11. Anthocyanin content of young leaves of seedlings grown in natural white light for one month. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

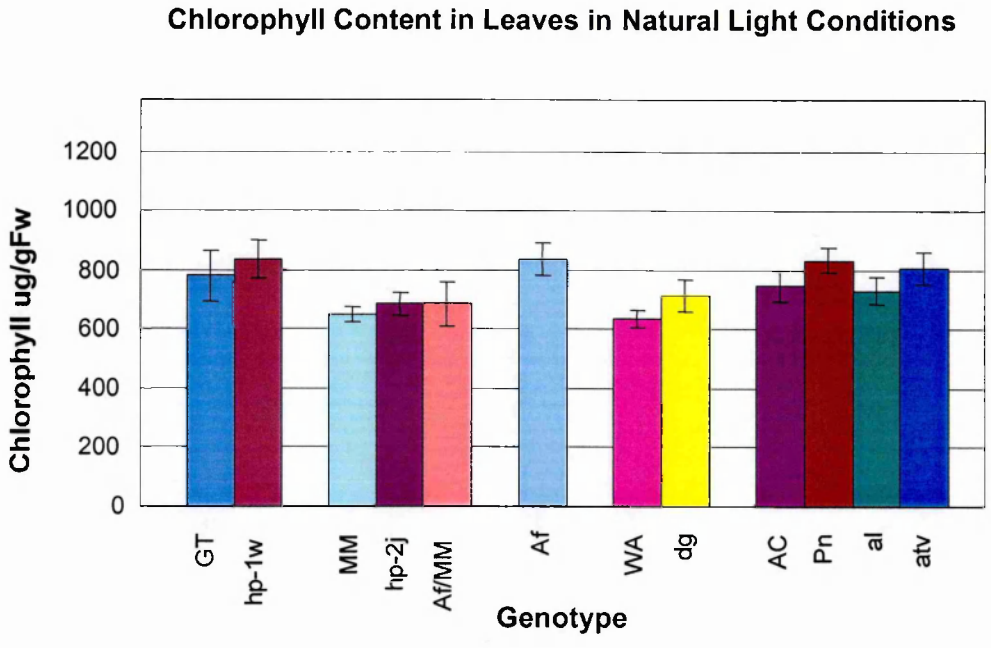


Figure 2.12. Chlorophyll content in young leaves of seedlings grown in natural white light for one month. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE

2.4.3 Anthocyanin Distribution and Chloroplast Development

To examine in which cell types anthocyanin pigments were located, hand-cut sections were made from roots, cotyledons and hypocotyls from selected mutant seedlings. Anthocyanin accumulates in the vacuole of plant cells, so taking sections for light microscopy was often a problem because the anthocyanin leaked out from cut cells. Furthermore, because hypocotyl cells of *hp* mutant seedlings were smaller than wild-type seedling hypocotyls, whereas the thickness of the sections was the same, quite some variability was observed. The light microscopy images shown are therefore illustrations of the trends observed. Anthocyanin was always found in the sub-epidermal layer of the hypocotyl and in the sub-epidermal layer below the main vascular system. However the location of anthocyanin in the trichomes differed, sometimes being present only in the basal cells, and sometimes also in the stalk cells.

2.4.3.1 high pigment2^{j}

Compared with wild type MM seedlings, the *hp2^{j}* mutant seedlings contain high levels of anthocyanin, especially in hypocotyls (Figure 2.13). The anthocyanin was visible in all cells of the sub-epidermal layer of the hypocotyl (Figure 2.13a and b). There was also a lot of anthocyanin below the vascular tissue of young leaves, whereas this was not the case in young leaves of MM seedlings (Figure 2.13c, d). The trichomes on the leaf edges sometimes contained anthocyanin in the basal cells and the first stalk cell. MM seedlings also developed anthocyanin in trichome basal cells and the first stalk cell, but concentrations were much lower (Figure 2.13e and f). Interestingly *hp2^{j}* mutant seedlings accumulated high levels of anthocyanin in the roots and the distribution was slightly different than in the hypocotyl. In roots the anthocyanin was observed in the cortex below the sub-epidermal cells (Figure 2.13h).

The chloroplasts of *hp2^{j}* mutant seedling cotyledons were a little larger compared to those in wild type seedlings. The *hp2^{j}* mutant seedlings developed fully mature chloroplasts in the roots when the plants were grown for 14 days in a 16h white light/8h dark regime (Figures 2.14d and 2.16b). The chloroplasts in the roots of *hp2^{j}* mutant seedlings have a comparable appearance to those in the cotyledons. MM seedling roots on the other hand contained only amyloplasts (Figures 2.14c and 2.16a).

2.4.3.2 *high pigment1*^{w}

In the hypocotyl of *hp1*^{w} mutant seedlings, a lot of anthocyanin was visible compared with wild type GT seedlings (similar to *hp2*^{j}, Figure 2.13). High amounts of anthocyanin were also visible in the sub-epidermal layer below the veins of young leaves. The trichomes on the leaf edges sometimes contained anthocyanin in the basal cells and the first stalk cell, whereas equivalent trichomes of GT seedlings contained low amounts of anthocyanin only in the basal cells but never in the stalk cells. Compared with GT seedlings, the anthocyanin concentration in *hp1*^{w} seedlings was always much higher, although the location of the anthocyanin was always the same (data not shown). The chloroplasts in *hp1*^{w} mutant seedling cotyledons were slightly larger compared to those in cotyledons from wild-type seedlings. More dramatically, however, *hp1*^{w} mutant seedlings also developed mature chloroplasts in the roots that were visually indistinguishable from the chloroplasts in the cotyledons (Figures 2.15d and 2.16c). In contrast, in GT seedling roots, only small amyloplasts developed (Figure 2.15c). In all these respects the *hp1*^{w} and *hp2*^{j} mutants were identical.

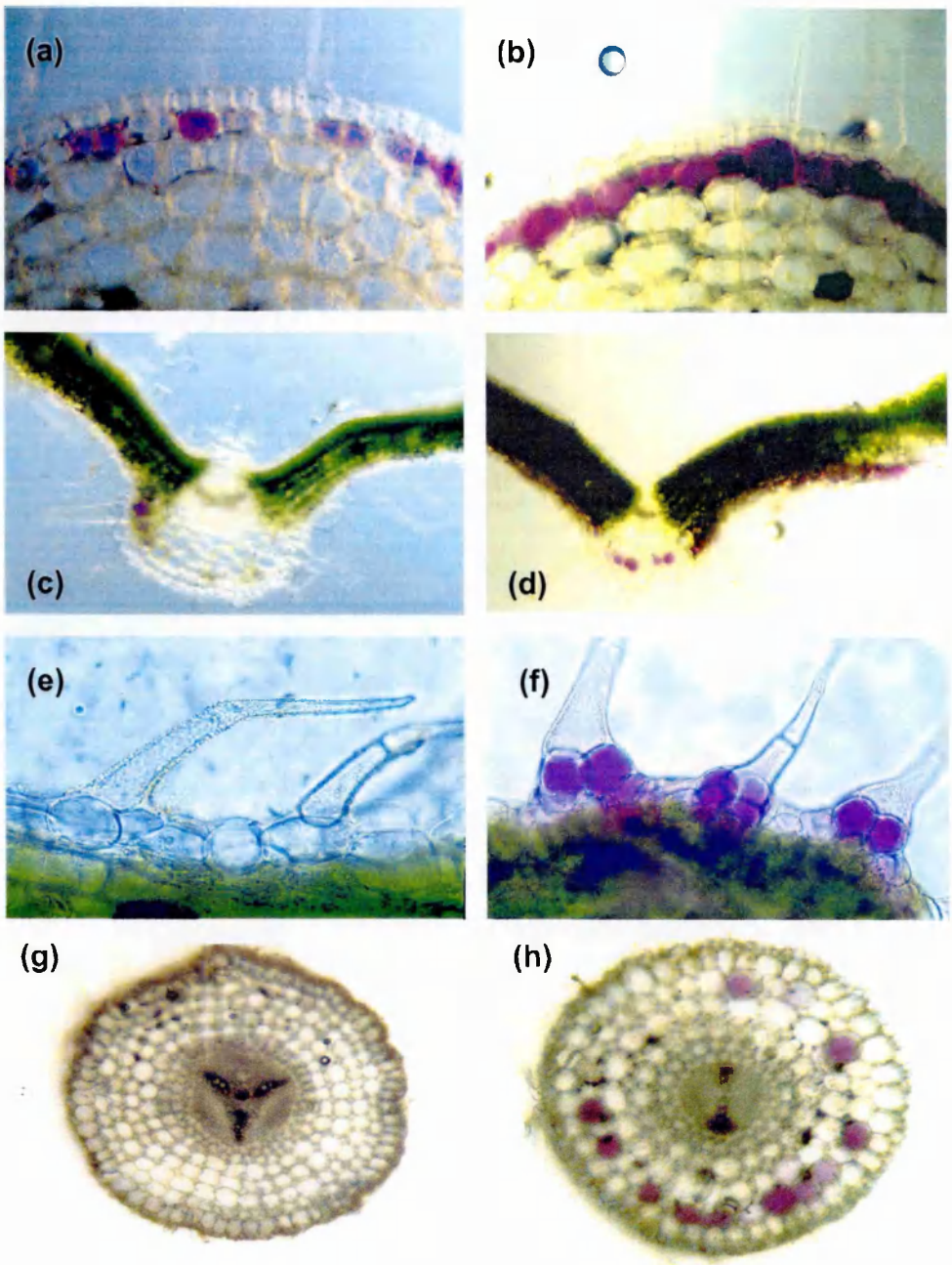


Figure 2.13. Anthocyanin distribution in different tissues of wild type Money Maker (MM) and *hp2* mutant seedlings. (a) and (b) Transverse sections of hypocotyls of MM and *hp2* seedlings. (c) and (d) Transverse sections of young leaves of MM and *hp2* seedlings. (e) and (f) Trichomes on the adaxial side of leaves of MM and *hp2* seedlings. (g) and (h) Transverse sections of roots of MM and *hp2* seedlings. In both wild-type MM and *hp2* mutants the anthocyanin was located in the sub-epidermal cells, but in *hp2* mutants the levels are much higher. Hand-cut sections were made from seedlings grown in natural white light conditions for one month.

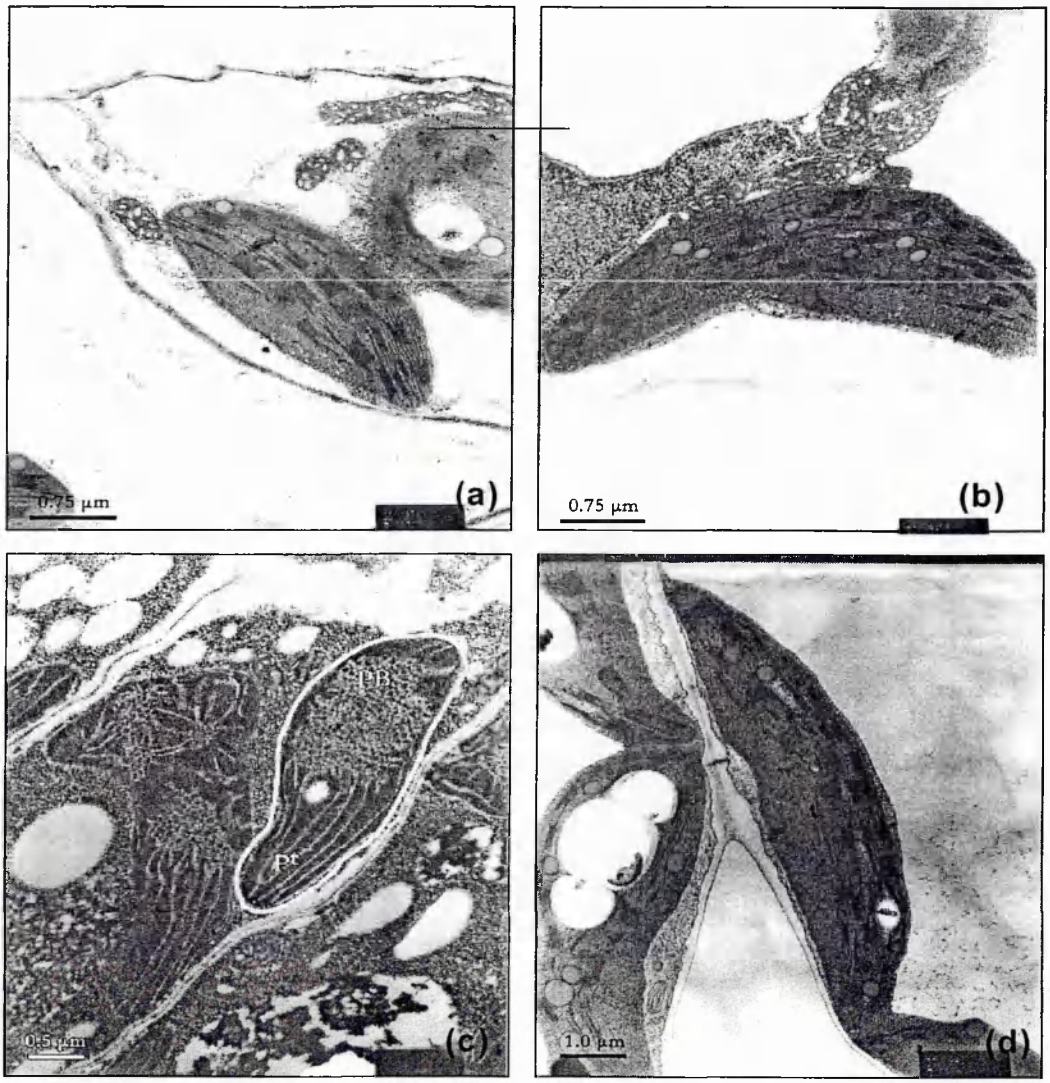


Figure 2.14. Chloroplast development in light grown 14 day old wild type MM and *hp2*^{j} seedlings. (a) and (b) Electron microscopic images from cotyledons of wild-type MM and *hp2*^{j} mutant seedlings.(c) and (d) Electron microscopic images from roots of wild-type MM and *hp2*^{j} mutant seedlings.

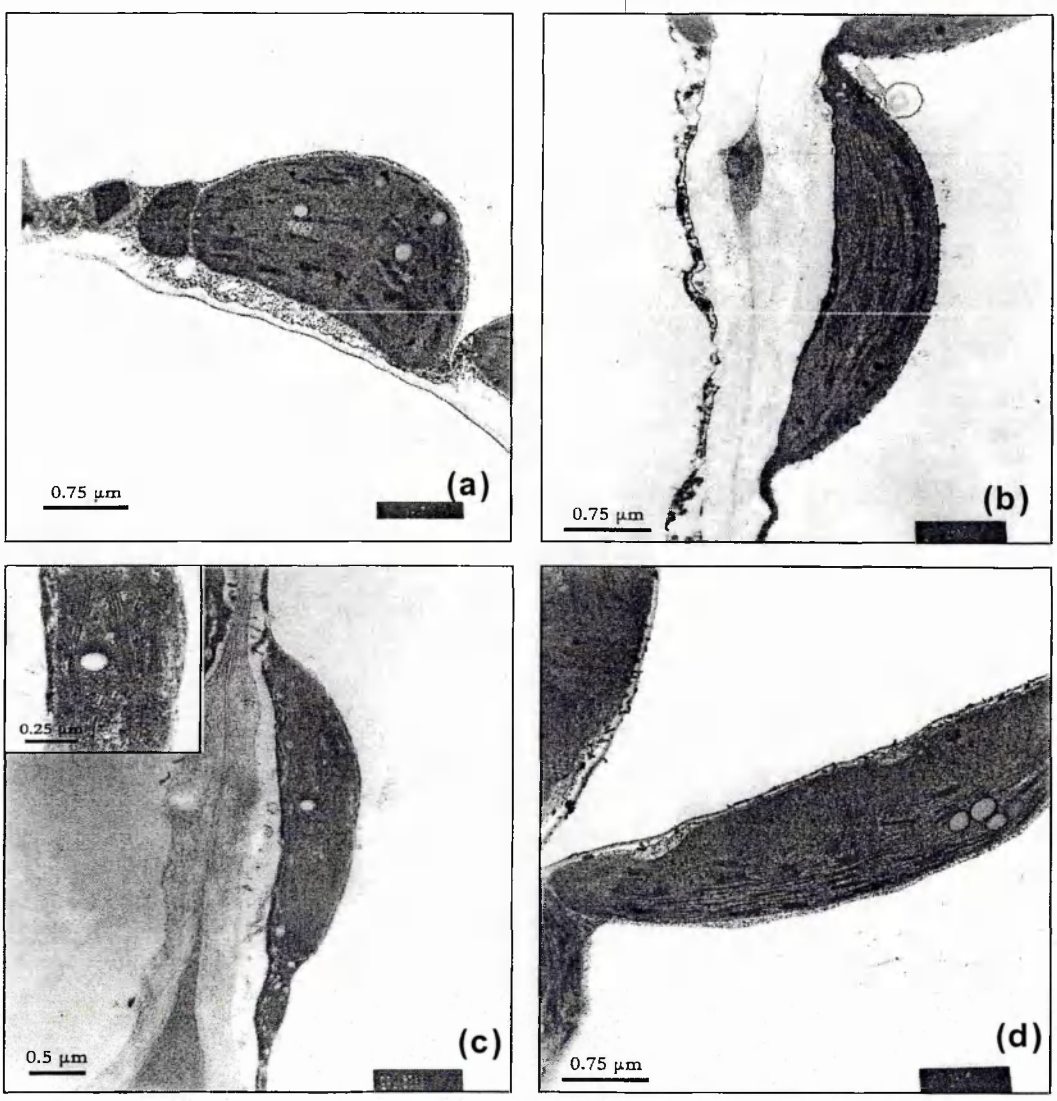


Figure 2.15. Chloroplast development in light grown 14 day old wild type GT and *hp1*^{w} seedlings. (a) and (b) Electron microscopic images from cotyledons of wild-type GT and *hp1*^{w} mutant seedlings. (c) and (d) Electron microscopic images from roots of wild-type GT and *hp1*^{w} mutant seedlings.

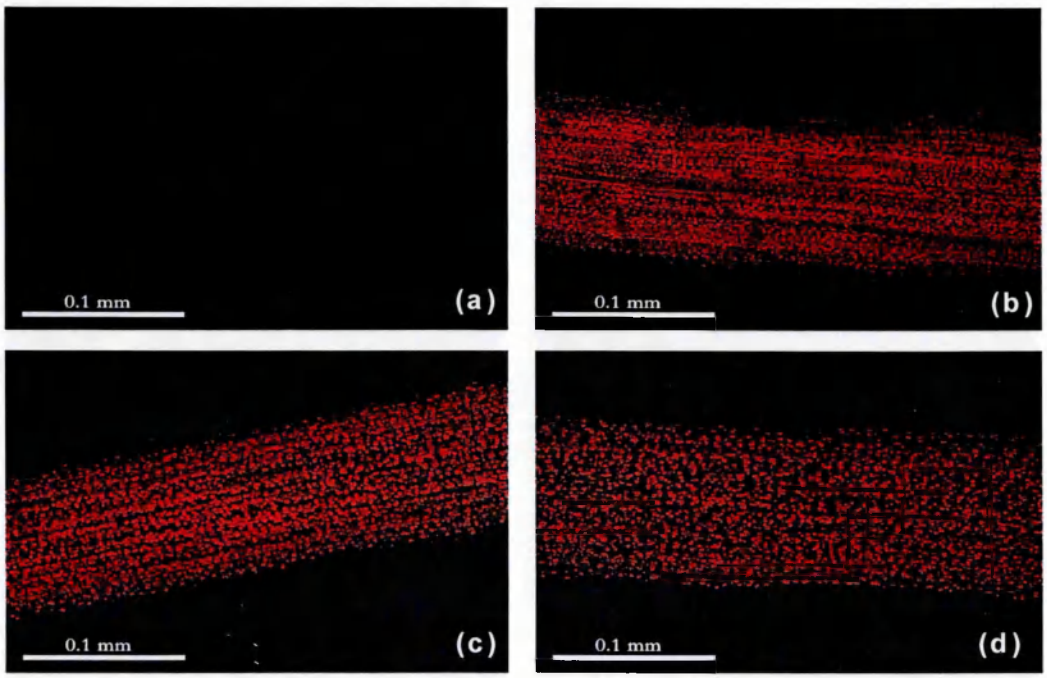


Figure 2.16. Plastid development in the roots of mutant seedlings roots grown in white light conditions. Laser Scanning confocal microscopy pictures representing roots from wild-type GT (a), *hp2*^{j} (b), *hp1*^{w} (c) and *atv* mutant seedlings roots contain chloroplats, visible as red dots. The seedlings were grown under artificial white light at 25 ^{0}C for 14 days in a 16-hour light and 8-hour dark photoperiod.

2.4.3.3 Anthocyanin fruit, Af/MM

The main characteristic of *Af* mutants is their dark purple fruit (Figure 2.3), but they also develop quite high levels of anthocyanin in the seedling hypocotyls (Figure 2.17). *Af* mutant seedlings also have a little anthocyanin in the sclerenchyme below the vascular tissue (Figure 2.17c, d). The location of the anthocyanin in the plant did not differ in *Af*/MM seedlings compared with MM seedlings. *Af*/MM seedlings have, in all parts of the plant studied, lower anthocyanin levels compared with the original *Af* mutant seedlings, but levels were nonetheless higher than in MM seedlings. The trichomes on the leaf edges contained higher anthocyanin in the basal cells and the first stalk cell compared to wild-type seedlings (Figure 2.17e, f). No plastid development or anthocyanin was observed in the roots of *Af* mutant seedlings.

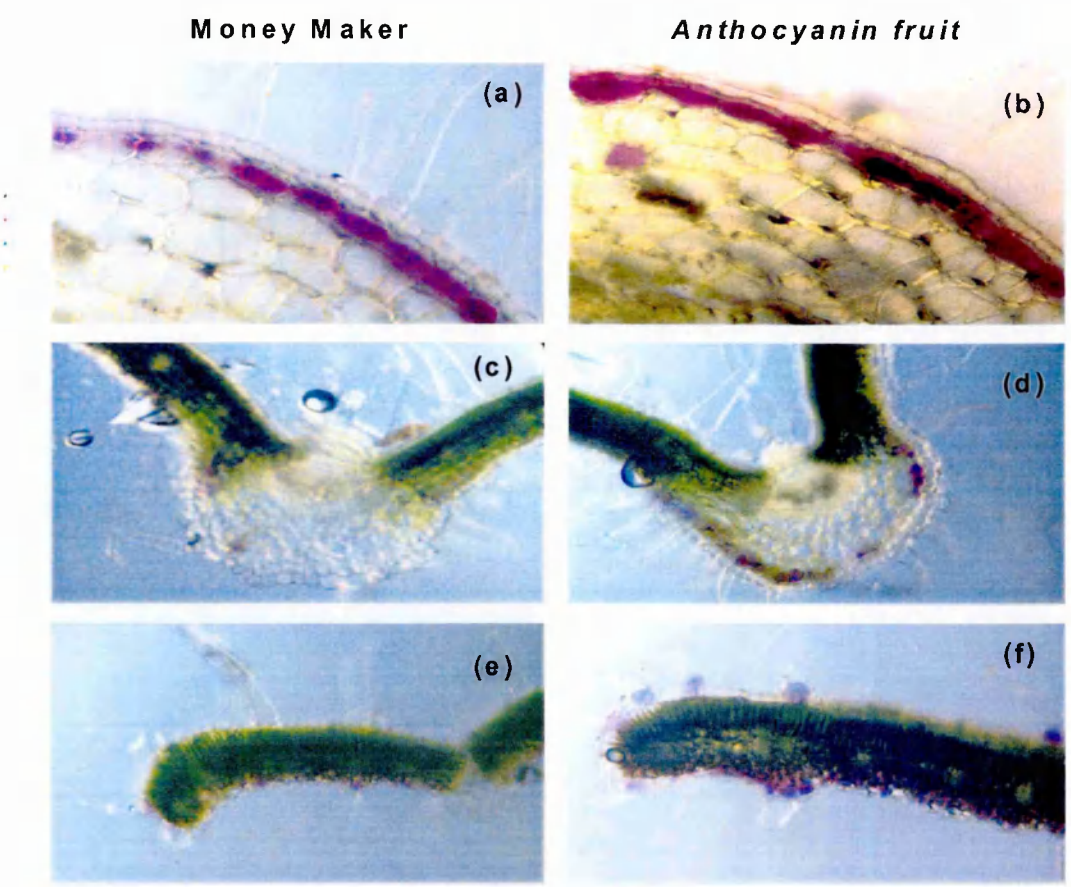


Figure 2.17. Anthocyanin distribution in different tissues of wild type Ailsa Craig (AC) and *Af* mutant seedlings. (a) and (b) Transverse sections of hypocotyls of AC and *Af* seedlings. (c) and (d) Transverse sections of young leaves of AC and *Af* seedlings. (e) and (f) Trichomes on the leaf edges of AC and *Af* seedlings. In both wild-type AC and *Af* mutants the anthocyanin was located in the sub- epidermal cells. However, in *Af* mutants the levels are slightly higher. In *Af* mutant leaves the anthocyanin was located mainly in the trichomes and the basal cells of the vascular tissue. Hand-cut sections were made from seedlings grown in natural white light for one month.

2.4.3.4 atroviolacea (atv)

Like the *hp* mutant seedlings, *atv* mutant seedlings contain high levels of anthocyanin compared to wild-type AC seedlings. In hypocotyls, leaf vascular tissue and trichomes, the anthocyanin concentration was much higher, but was always in the same location (Figure 2.18). In the trichomes anthocyanin was located in the basal cells and also the stalk cells (Figure 2.18f). The lower part of the leaf edges contained high levels of anthocyanin (Figure 2.18d). Like the *hp* mutant seedlings *atv* mutant seedlings develop slightly larger chloroplasts in the cotyledons compared to wild-type seedlings and *atv* mutant seedling roots also develop mature chloroplasts in light-grown plants (Figure 2.16d, 2.19d) whereas AC seedlings do not. However, no anthocyanin was observed in the roots of *atv* mutant seedlings.

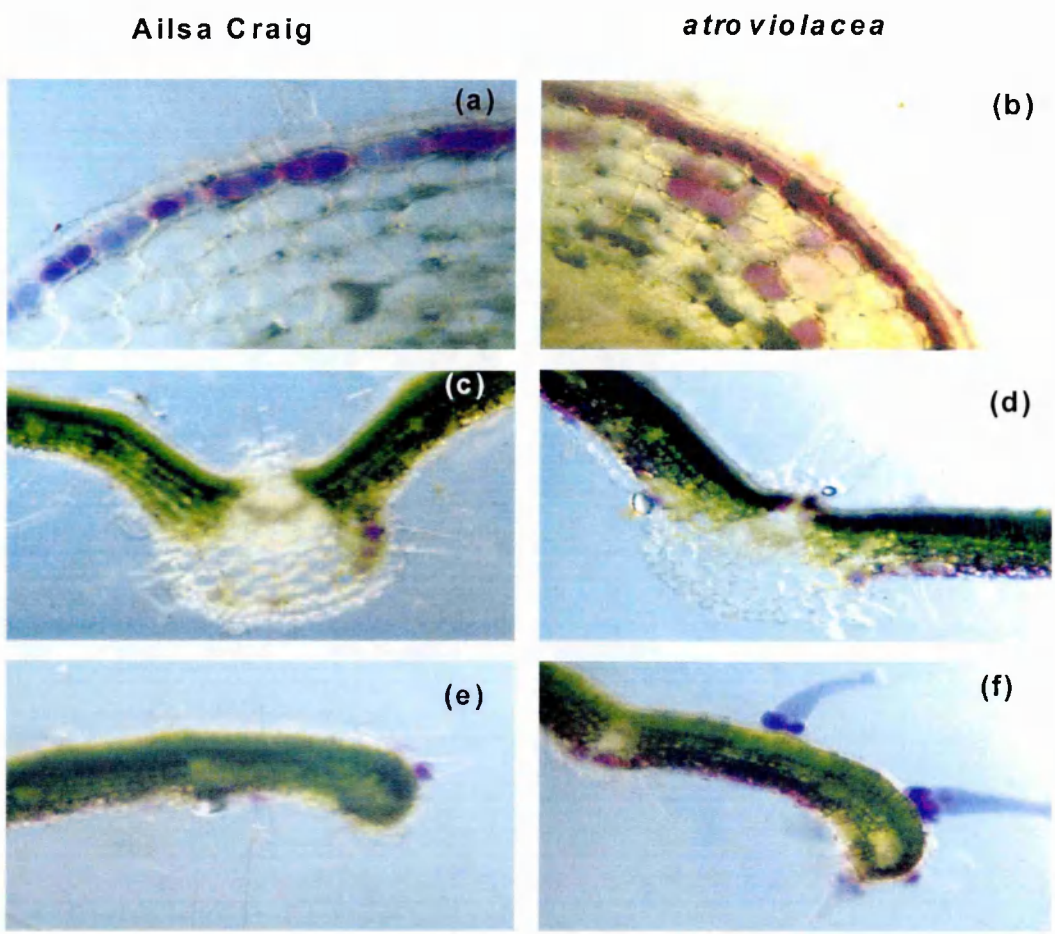


Figure 2.18. Anthocyanin distribution in different tissues of wild type Ailsa Craig (AC) and *atv* mutant seedlings. (a) and (b) Transverse sections of hypocotyls of AC and *atv* seedlings. (c) and (d) Transverse sections of young leaves of AC and *atv* seedlings. (e) and (f) Trichomes on the leaf edges of AC and *atv* seedlings. In both AC and *atv* mutants the anthocyanin was located in the sub-epidermal cells, however in *atv* mutants the levels are much higher. In *atv* mutants the anthocyanin on the leaves was located mainly in the basal cells and in trichomes. Hand-cut sections were made from seedlings grown in natural white light for one month.

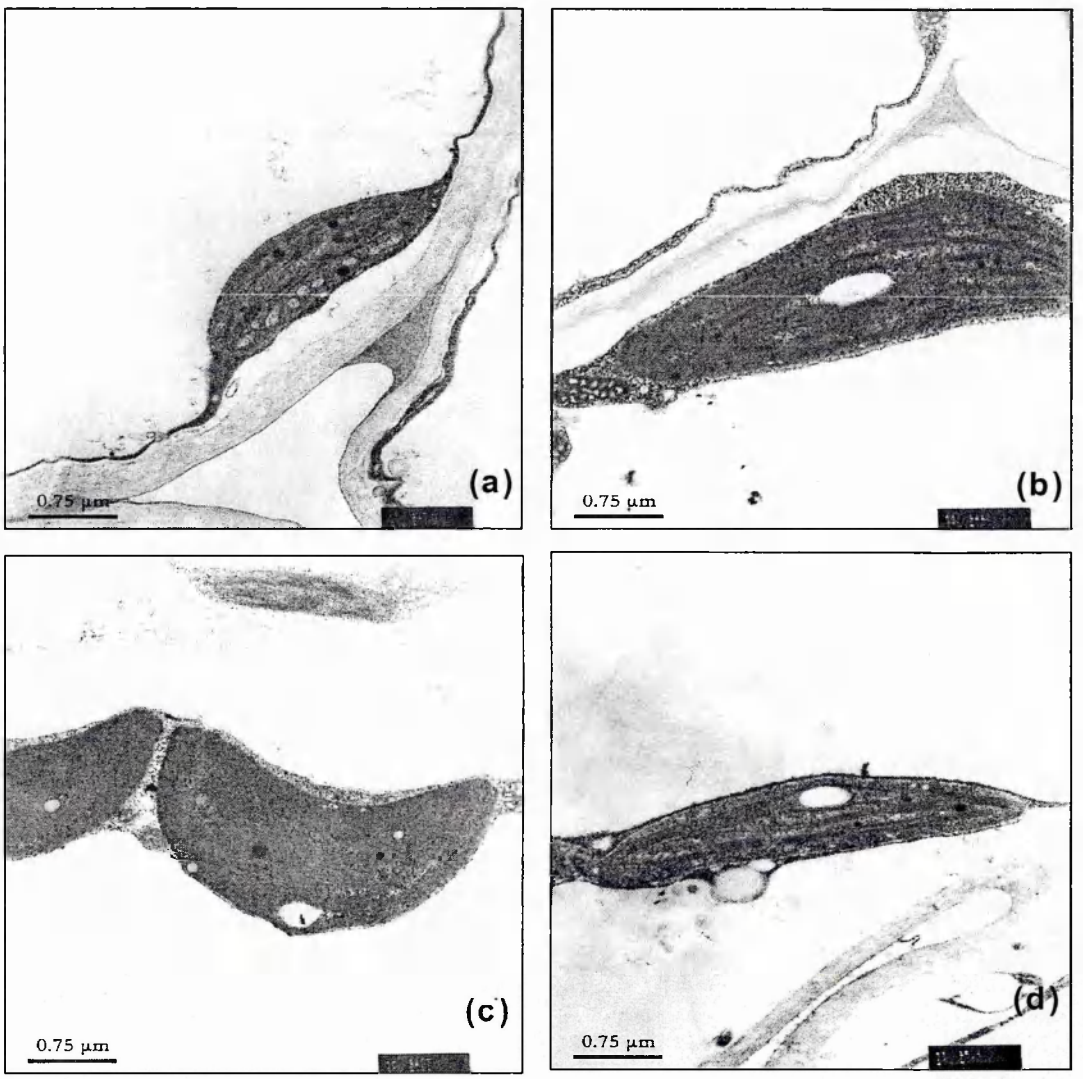


Figure 2.19. Chloroplast development in light grown 14 day old wild type AC and *atv* seedlings. (a) and (b) Electron microscopic images from cotyledons of wild-type AC and *atv* mutant seedlings. (c) and (d) Electron microscopic images from roots of wild-type AC and *atv* mutant seedlings.

2.4.3.5 Punctate (Pn)

Pn mutant seedlings did not differ from the wild type seedlings, except for the trichomes, all of which contained anthocyanin in the basal cells and in all the stalk cells (data not shown), similar to the trichomes of *atv* mutant seedlings (Figure 2.13).

2.4.3.6 dark green (dg)

In terms of anthocyanin distribution, *dg* seedlings did not differ from wild-type WA seedlings. Only the dark green shoulder of the immature fruits of this mutant (Figure 2.4) was different from fruits from wild type plants (data not shown).

2.4.4 Development of Plastids in the Dark

In Arabidopsis, the *cop/det/fus* mutants display characteristics of light-grown plants when grown in complete darkness, such as reduced hypocotyl length and partial plastid development. The *hp2* tomato mutant is mutated in the tomato homologue of Arabidopsis *DET1*, although it does not display any visible phenotypes in the dark (Mustilli et al., 1999). The only characteristic observed was that of partial plastid development in dark grown *hp2* mutant seedling cotyledons. Plastid development in darkness of selected tomato mutant seedlings was therefore studied. Hypocotyl length of dark-grown seedlings was also measured to confirm whether it was affected in these mutants.

As previously reported (Mustilli et al., 1999), when $hp2^{j}$ mutant seedlings were grown in complete darkness, plastids in the cotyledons were slightly more developed compared to those in wild-type seedlings. The prolamellar bodies of the etioplasts were a little smaller and there were more prothylakoid membranes protruding from them (Figure 2.19a, b).

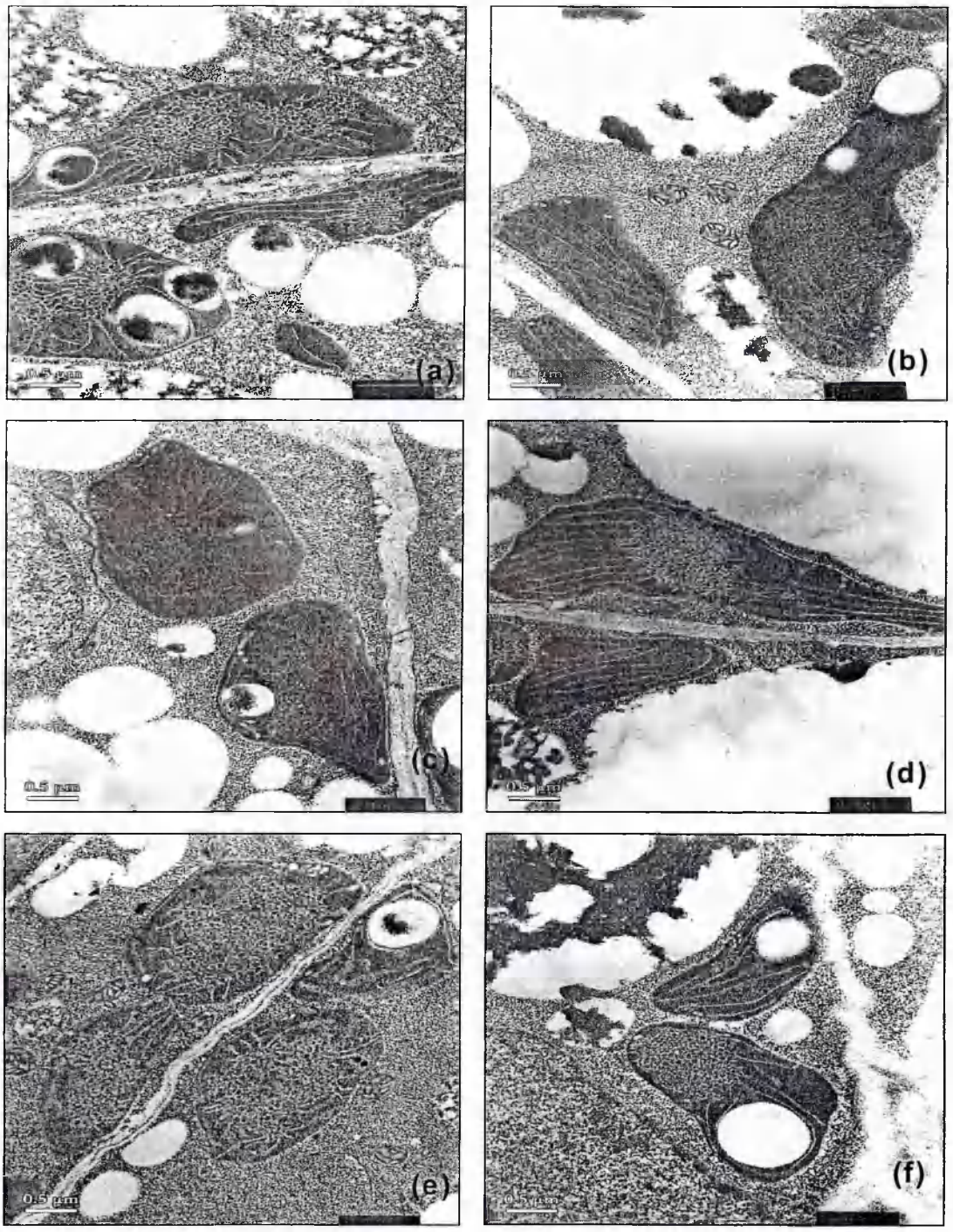


Figure 2.20. Plastid development in mutant seedlings grown in complete darkness. (a) and (b) Electron microscopic images from cotyledons of wild-type MM and *hp2*^{j} mutant seedlings. (c) and (d) Electron microscopic images from cotyledons of wild-type GT and *hp1*^{w} mutant seedlings. (e) and (f) Electron microscopic images from cotyledons of wild-type AC and *atv* mutant seedlings.

In dark-grown *hp1*^{w} mutant seedlings, the plastids were also more developed than in dark-grown wild type seedlings. The majority of plastids in GT wild-type seedlings were typical etioplasts containing large prolamellar bodies with very few prothylakoid membranes. On the contrary *hp1*^{w} mutant cotyledons contained more plastids with smaller prolamellar bodies and more prothylakoid membranes (Figure 2.19c, d).

Like the *hp* mutant seedlings, in *atv* mutant seedlings grown in the dark, cotyledons contained more partially developed plastids, with smaller prolamellar bodies and more prothylakoid membranes (Figure 2.19e, f).

The other studied mutants did not show any alterations in plastid development (data not shown).

2.4.5 Continuous Broad-band Light Experiments

To better understand the phenotypes of mutant seedlings we performed continuous broadband experiments using blue, red and far red light. At the same time seedlings were also grown in complete darkness for the same period of time and analyzed. In this study we only used the *hp*, *atv* and *Af* mutants because of their stronger responses found in the above-described white light experiments. The *atv* mutants used in this study had been backcrossed to GT 4 times.

In continuous blue light the hypocotyl length of *hp1*^{w} mutant seedlings was a little shorter than wild-type GT seedlings, whereas hypocotyl length of *atv* mutant seedlings was similar to GT seedlings (Figure 2.21a). The hypocotyl lengths of both *hp2*^{j} and *Af* mutant seedlings were almost half the size of the wild-type Money Maker seedlings (Figure 2.21a). Concerning anthocyanin content of hypocotyls, *hp1*^{w} mutant seedlings showed the highest anthocyanin accumulation (3-times more than GT seedlings) but only slight or negligible increases were observed in the other mutant seedlings (Figure 2.21b). Chlorophyll content of these mutants in continuous blue light is essentially the same in all genotypes (Figure 2.21d). From these results it is evident that both *hp* mutants are much more responsive to continuous blue light than the other mutants, as well as *Af* mutant seedlings, in which a reduction in hypocotyl length was also observed.

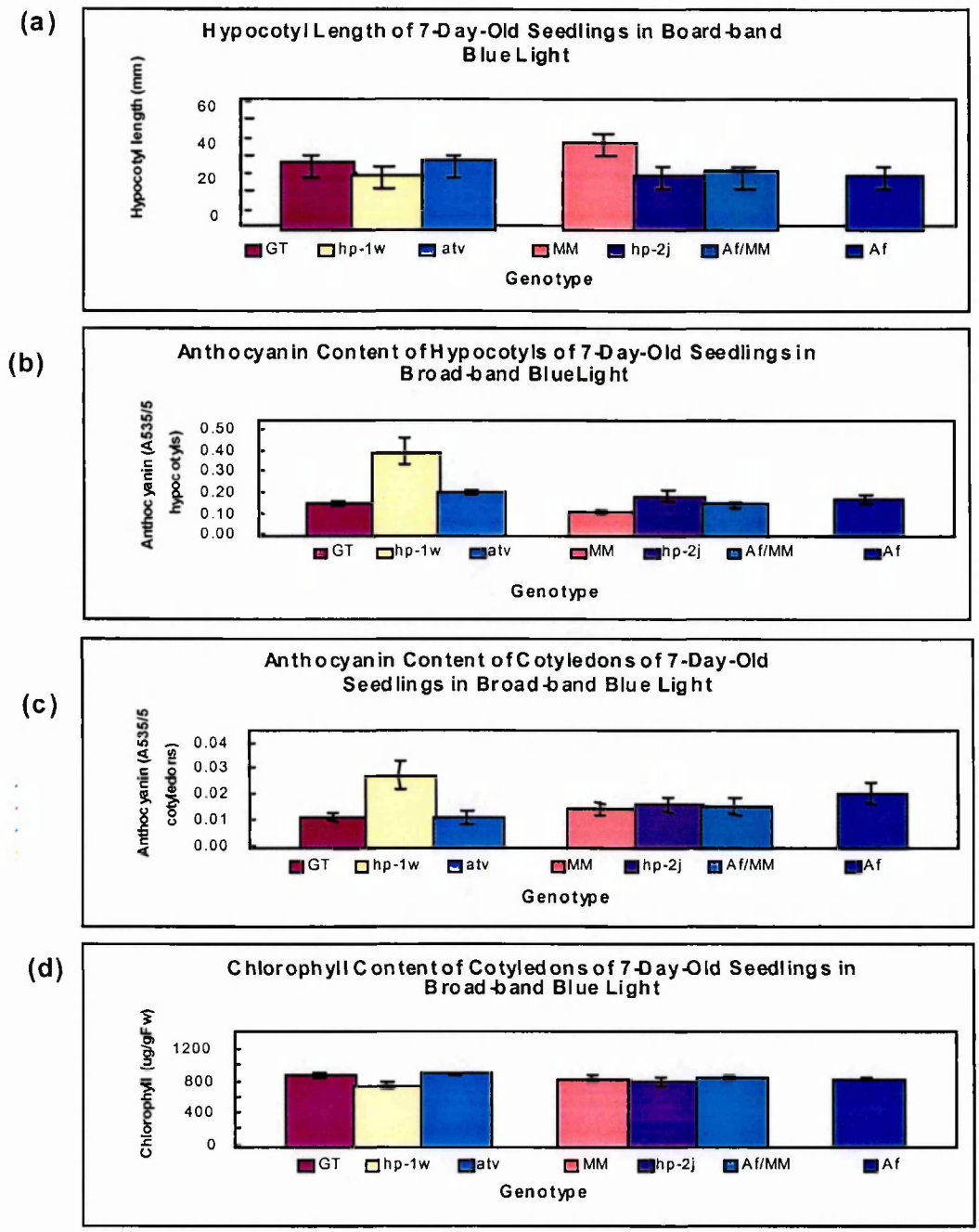


Figure 2.21. Changes in hypocotyl length, anthocyanin content and chlorophyll content in mutant seedlings and respective wild-type seedlings grown in continuous blue light at 25 ^{0}C for 7 days. (a) Hypocotyl lengths of seedlings. (b) Anthocyanin content of hypocotyls of seedlings. (c) Anthocyanin content of cotyledons of seedlings. (d) Chlorophyll content of cotyledons of seedlings. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

In continuous red light, the hypocotyl length of *hp1*^{w} mutant seedlings was shorter than in wild-type seedlings, whereas the hypocotyl length of the *hp2*^{j} mutant seedlings was significantly shorter than in wild-type MM seedlings. However, the hypocotyl lengths of the other mutant seedlings were only slightly shorter (Figure 2.22a). Anthocyanin content in hypocotyls of *hp1*^{w} seedlings was 5-times higher than in wild-type GT seedlings (Figure 2.22b), whereas in cotyledons it was 4-times higher (Figure 2.22c). Similar trends were observed in *hp2*^{j} mutant seedlings, but were lower than in *hp1*^{w} mutant seedlings. In the other mutant seedlings the anthocyanin content of both hypocotyls and cotyledons was only slightly higher than in wild-type material (2.22b, c). Chlorophyll content of both *hp* mutants is slightly lower than in wild type seedlings, whereas both *atv* and *Af* mutant seedlings displayed similar levels as in cotyledons from wild-type seedlings (Figure 2.22d).

In continuous far red light both *hp* mutants died within 4 days after germination. The hypocotyl length of *atv* mutant seedlings was similar to wild-type GT seedlings, whereas the hypocotyl length of *Af* seedlings was slightly shorter than in MM seedlings (Figure 2.23a). *atv* mutant seedlings showed slightly elevated levels of anthocyanin in hypocotyls compared to wild-type GT seedlings, whereas *Af* mutant seedlings were similar to wild-type seedlings (Figure 2.23b). The anthocyanin content of cotyledons from *Af* and *atv* mutant seedlings was similar to that found in wild-type seedlings (Figure 2.23c). Chlorophyll content was negligible in all cases (Figure 2.23d).

In dark experiments no significant differences were observed, except that hypocotyl length of *hp* and *Af* mutant seedlings was shorter than their wild-type seedlings (Figure 2.24a). No major differences in anthocyanin and chlorophyll content between mutant seedlings and wild type seedlings were observed (Figure 2.24b, c, d).

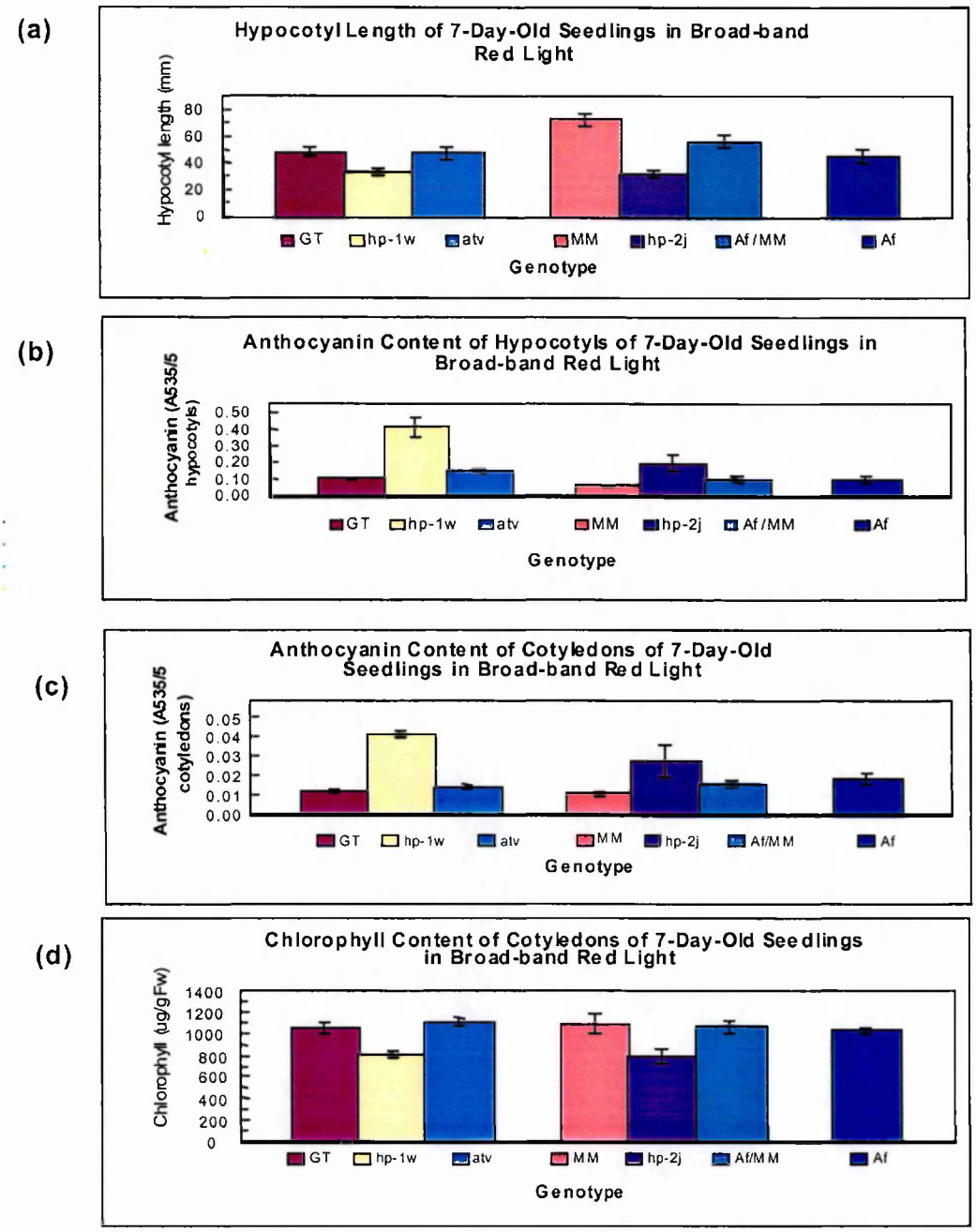


Figure 2.22. Changes in hypocotyl length, anthocyanin content and chlorophyll content in mutant seedlings and respective wild-type seedlings grown in continuous red light at 25 ^{0}C for 7 days. (a) Hypocotyl lengths of seedlings. (b) Anthocyanin content of hypocotyls from seedlings (c) Anthocyanin content in cotyledons of seedlings. (d) Chlorophyll content of cotyledons of seedlings. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE.

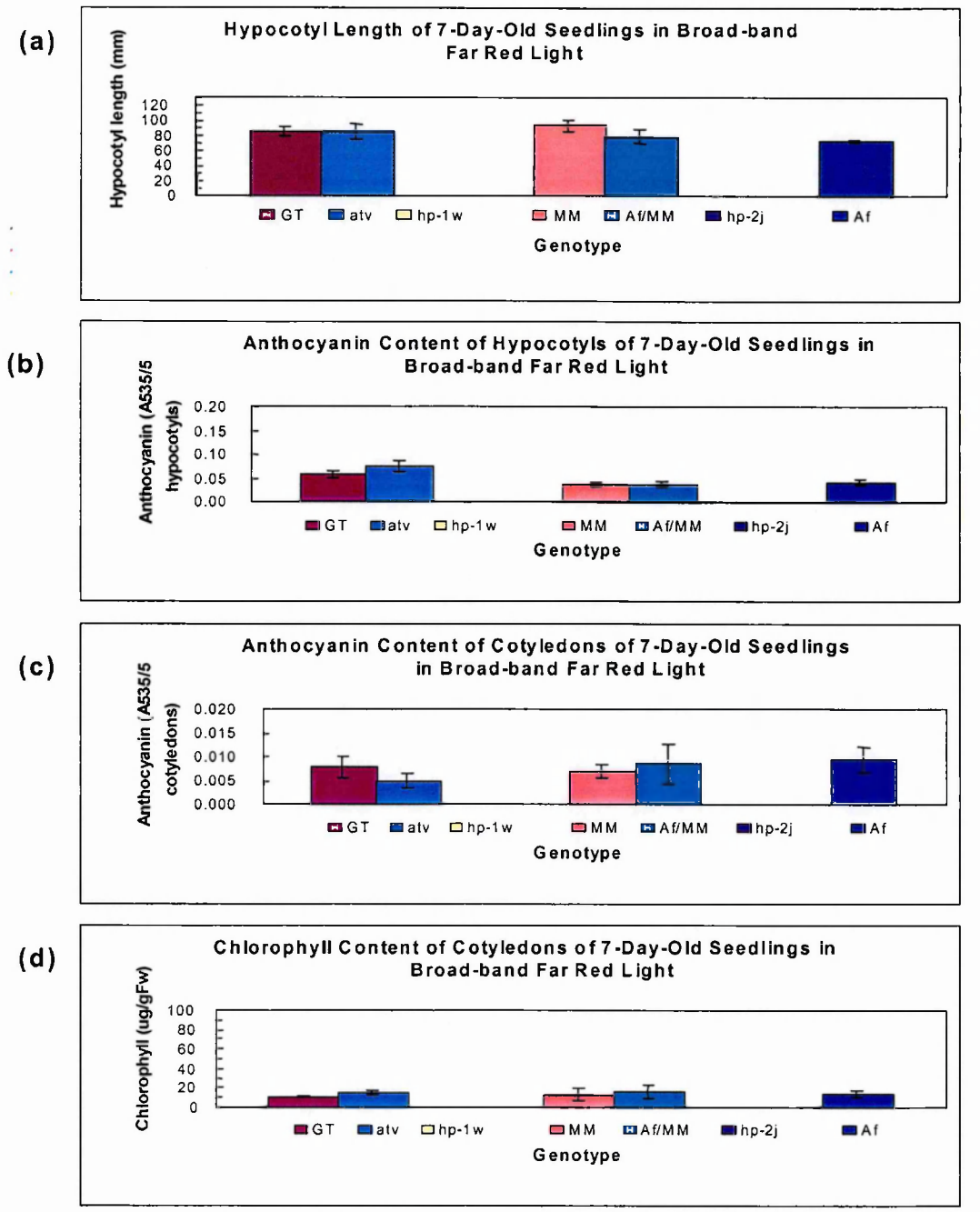


Figure 2.23. Changes in hypocotyl length, anthocyanin content and chlorophyll content in mutant seedlings and respective wild type seedlings grown under continuous far red light at 25 ^{0}C for 7 days. (a) Hypocotyl lengths of seedlings. (b) Anthocyanin content of hypocotyls of seedlings. (c) Anthocyanin content in cotyledons of seedlings. (d) Chlorophyll content in cotyledons of seedlings. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE. In (d) the scale bar has been enlarged to better visualize the data.

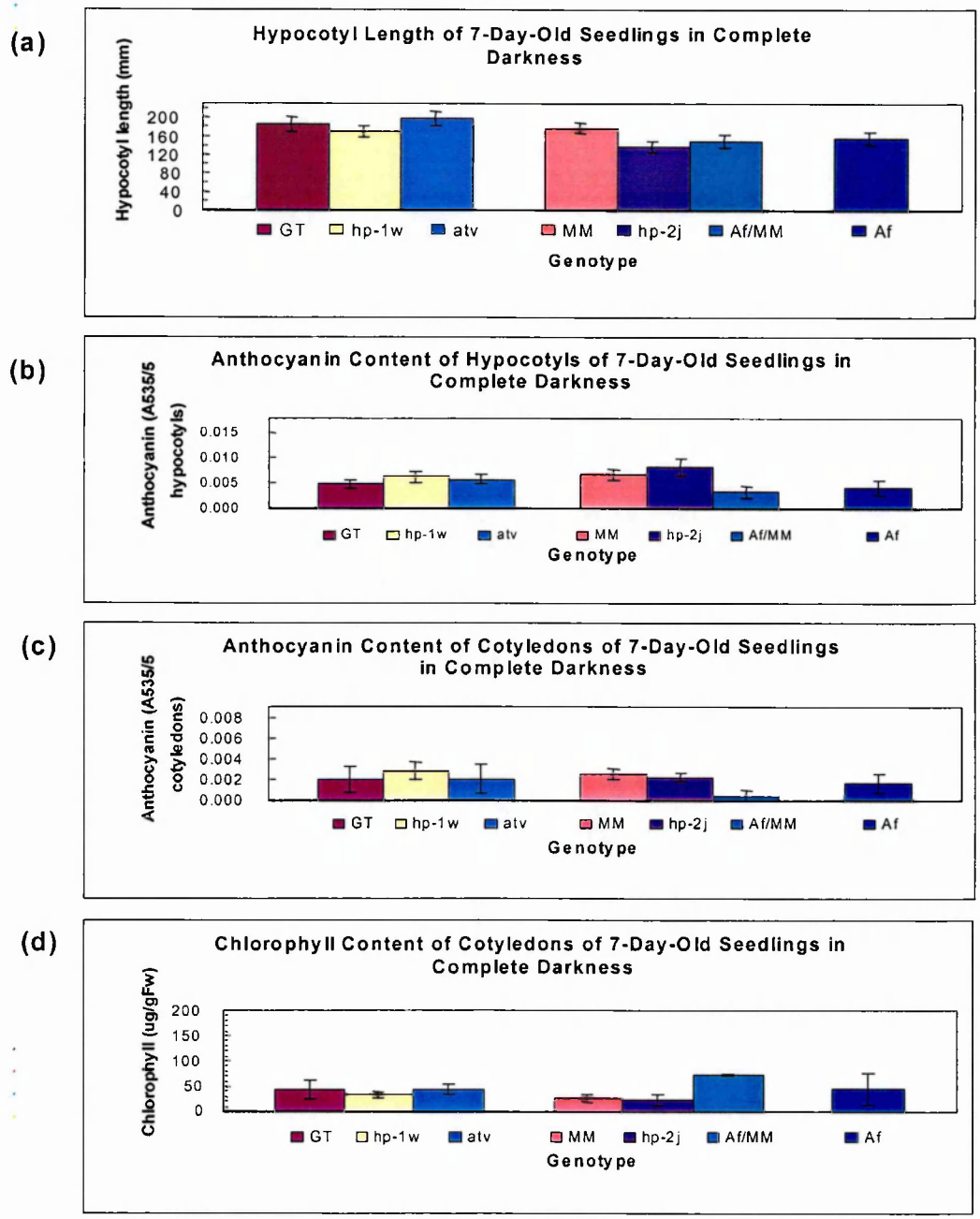


Figure 2.24. Changes in hypocotyl length, anthocyanin content and chlorophyll content in mutant seedlings and their respective wild types grown under continuous darkness at 25 ^{0}C for 7 days. (a) Hypocotyls lengths of seedlings. (b) Anthocyanin content of hypocotyls of seedlings. (c) Anthocyanin content of cotyledons of seedlings. (d) Chlorophyll content of cotyledons of seedlings. Values are the means of 5 seedlings from a single representative experiment, and the experiments were repeated two times. Error bars indicate ±SE. In (d) the scale bar has been enlarged to better visualize the data.

2.4.6 Light-Regulated Gene Expression in White Light

To examine the effects of these mutations on gene expression, northern blot analysis to examine light-regulated gene expression using *CHS* (chalcone synthase) and *CAB* (chlorophyll *a/b* binding protein) gene fragments as probes was performed. Seedlings of each of the mutants, together with their respective wild-types, were grown in soil for 5 days in the dark at 25 ^{0}C, and were then transferred to white light. Samples were collected for RNA extraction at 0, 3, 6, 9 and 12 hour intervals as described in Materials and Methods. The 0 hour samples were collected in green safe light. The expression levels of *CHS* in *Af* mutants were slightly higher than in wild-type seedlings in the dark (Figure 2.25). A significant difference in *CHS* gene expression levels in white light was also observed in all the four mutants compared with their wild-type plants. Like the *hp* mutants, *atv* and *Af* mutants also displayed elevated levels of *CHS* mRNA in white light. Surprisingly, *Af* has higher levels of *CHS* expression in the dark whereas in *atv CHS* levels in the dark were almost similar to wild-type levels (Figure 2.25). In case of *dg* no significant differences were observed in both *CAB* and *CHS* expression levels compared to the wild-type WA seedlings. The other mutants did not differ significantly from their respective wild-types (data not shown).

CAB gene expression was slightly elevated in $hp1^{w}$, $hp2^{j}$, *atv* and *Af* mutants in white light. In the dark both *hp* mutants showed slightly elevated levels of *CAB* expression compared to wild-type seedlings (Figure 2.25). In both *Af* and *atv* mutants, *CAB* expression levels were similar to their respective wild type seedlings.

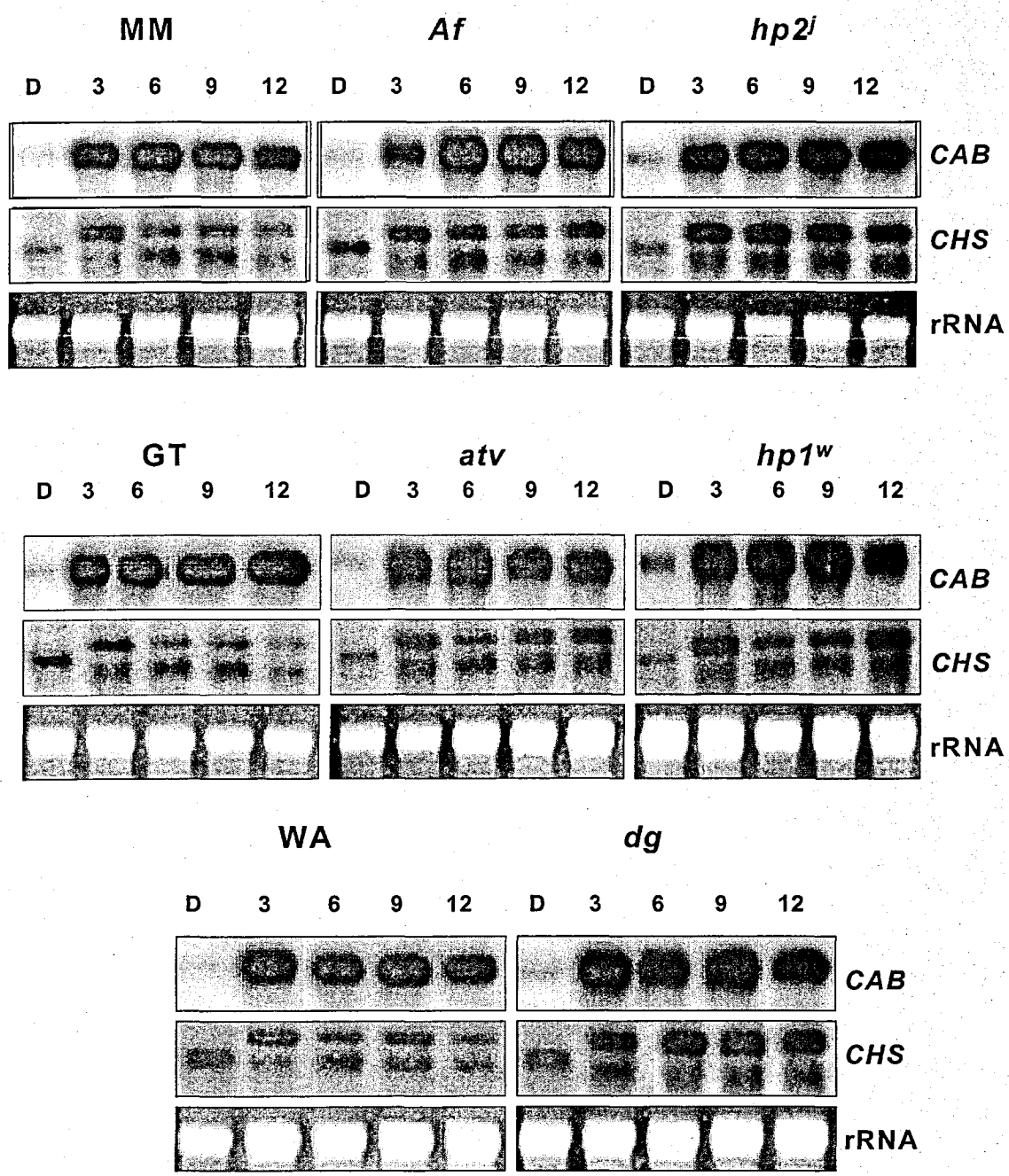


Figure 2.25. Gene expression in different mutants and respective wild-type seedlings. Seedlings were grown at 25 ^{0}C for 5 days in the dark and were transferred to white light. Samples were collected at 0, 3, 6, 9 and 12-hour intervals. RNA was extracted from the cotyledons and apical hooks of mutant and wild-type seedlings. 10 µg of total RNA was loaded and analyzed for expression of *CAB6* and *CHS1* genes after RNA gel blotting. 28S rRNA is shown as a loading control.

2.5 Discussion

In this study, several light hyper-responsive mutants of tomato have been characterized, with the aim of determining their basic photobiological characteristics. Specifically, the mutant collection included the *high pigment* (*hp1* and *hp2*), *atroviolacea* (*atv*), *Anthocyanin fruit* (*Af*), *Punctate* (*Pn*), *anthocyaninless* (*al*) and *dark green* (*dg*) mutants. These mutants have all been described several years ago (see 2.2) but only the *high pigment* mutants have been characterized at the molecular level. For mutant characterization several light conditions were used, including artificial white light in laboratory conditions, white light in natural greenhouse conditions, continuous broadband light (blue, red, far/red light) and complete darkness. Most previous studies were conducted in laboratory conditions, so the inclusion of natural greenhouse conditions allowed additional characterization. For example, during the low temperatures of January anthocyanin content was much higher than in the experiments conducted in November, when the temperatures were slightly higher. The results presented indicate that the *high pigment* (*hp1* and *hp2*), *atv* and *Af* mutants were the most interesting in terms of their photoresponse characteristics and may encode important light signal transduction intermediates. The other mutants did not show readily understandable defects in any of the light conditions used here.

Nonetheless, it is interesting to note that trichromes on *Pn* mutant leaves showed higher anthocyanin accumulation and that the *al* mutant seedlings showed decreased anthocyanin levels soon after germination, because *Pn* and *al* have been proposed to be allelic to each other (Rick, 1968). Such opposing phenotypes in dominant and recessive alleles at the same locus may indicate that the affected gene encodes a regulator of anthocyanin biosynthesis.

Similar to *Pn* mutants, *dg* mutant seedlings did not display exaggerated photoresponses in any of the light conditions used here. However the dark green fruit phenotypes of these mutants were intermediate between wild-type and *hp* fruits, in that *dg* fruits displayed only a dark green shoulder. Notwithstanding, other work in the laboratory suggests that this *dg* allele is not an additional allele of *hp1* or *hp2*, in contrast to the *dg (Manapal)* mutant, which was found by Levin et al. (2002) to be allelic to *hp2*.

Over the past few years the *high pigment* mutants have been particularly well studied (reviewed in Kendrick et al., 1997). Van Tuinen et al. (1997) confirmed that the *hp1* and *hp2* alleles represent different genes and called the loci *HP1* (previously *HP*) and *HP2*. Peters et al. (1992) suggested that the *hp1* mutation modifies a basic process affecting photomorphogenesis on the basis of the pleiotropic phenotypes of *hp1* mutants. On the basis of the recessive nature of the mutation they proposed that phytochrome action is under the constraint of the *HP1* gene product. Similar hypotheses were made about the *HP2* gene product, and gene cloning has recently confirmed this, by showing that these gene products encode, respectively, DDB1 and DET1, important regulators of photomorphognesis in *Arabidopsis* (Mustilli et al., 1999; Levin et al., 2002; Lieberman et al., 2004; Liu et al., 2004).

Some of the results presented here confirmed previous findings about the *high pigment* mutants. For example, the increased anthocyanin content of *hp* mutant hypocotyls and cotyledons is well documented (Peters et al., 1989; Kerkhoffs and Kendrick, 1997; Kerckhoffs et al., 1997[b]; van Tuinen et al., 1997 and Mustilli et al., 1999), as is plastid development in cotyledons of dark-grown seedlings (Mustilli et al., 1999). However, the more detailed study conducted here in continuous broadband experiments showed that both *hp* mutants differed slightly from their behaviour in white light experiments. In continuous blue and red light the hypocotyl length of $hp2^{j}$ seedlings was markedly shorter than wild-type seedlings, whereas the hypocotyl length of $hp1^{w}$ seedlings was only slightly shorter. However the anthocyanin content of $hp1^{w}$ seedlings (both hypocotyls and cotyledons) was much higher than $hp2^{j}$ seedlings, even when taking into account the differences due to their respective wild-type backgrounds. These results support the proposal that *hp1* mutants exhibit a strong amplification of HIR under broadband light conditions (Kerckhoffs et al., 1997[b]), and indicate that this is also the case for *hp2* mutants, albeit for a different response (hypocotyl growth inhibition rather than anthocyanin accumulation). The stronger effect of the *hp2* allele on hypocotyl and stem length was also found in the white light and natural light experiments (see Figs 2.5 and 2.9), although the increased anthocyanin accumulation in *hp1* mutants compared with *hp2* mutants was only reproduced in natural light conditions and not in white light (see Figs 2.5 and 2.9). Multiple photoreceptors can control hypocotyl elongation, although with distinct kinetics. In *Arabidopsis*, phytochrome A and B, the blue light receptor *cry1* and a genetically separable UV-A receptor have been shown to contribute to the inhibition of hypocotyl cell elongation (Koornneef et al., 1980). The results obtained here clearly indicate

that the *HP1* and *HP2* gene products are likely to be acting under several photoreceptors, although the responses are not equal.

The most interesting finding from the broadband light experiments was that both *hp* seedlings died in continuous far red light (Fig. 2.23). Barnes et al. (1996) previously showed a similar phenotype in *Arabidopsis*, a block of greening in *phyA* mutants following exposure to white light after far red light treatment. Disrupted phyA signalling was found to be the cause for this effect, and so it is likely that increased phyA signalling in the *hp* mutants is responsible for this death phenotype. It will be of interest to further examine this phenomenon, by studying other phyA responses in far red light, such as inhibition of *protochlorophyllide oxidoreductase* (*POR*) expression, because Barnes et al. (1996) presented evidence that a miscoupling of phytochrome-regulated POR expression led to photooxidation in the phyA mutants, due to misregulated protochlorophyllide accumulation.

Another interesting finding from the experiments reported here concerns the phenotypes of *hp* mutant roots. The roots from light-grown *hp* mutants were found to display chloroplast-like plastids containing thylakoid membrane systems rather than the amyloplasts typically found in wild-type root cells, and to contain significant amounts of anthocyanin (Fig. 2.14 and 2.15). These results highlight the highly deregulated nature of the photoresponses in these mutants, because chloroplasts and anthocyanin are not usually present in wild-type tomato roots.

By contrast, increased anthocyanin and chloroplast-derived pigmentation are well known aspects of the *hp* mutant phenotypes (Peters et al., 1998). To study this at the molecular level, expression analysis of light regulated genes, encoding a *chlorophyll a/b-binding* protein (*CAB*) and *chalcone synthase* (*CHS*) was performed in white light grown seedlings. In tomato the expression of both *CAB* and *CHS* genes has been found to be controlled by phytochrome and cryptochromes (Sharrock et al., 1988). In both *hp* mutant seedlings only *CHS* levels were found to be upregulated compared with wild-type seedlings (Fig. 2.25). In contrast, dark grown *hp* mutant seedlings accumulate higher levels of *CAB* transcripts than wild-type seedlings. Similar results were reported by Peters et al. (1998), but only for *hp1* mutant seedlings. These results suggest that *CAB* gene expression in response to light is not markedly affected by the *hp* mutations, perhaps inferring that these mutations affect plastid compartment size (either number of plastids per cell or plastid size), as proposed for *hp1* by

Cookson et al. (2003). Notwithstanding, the *hp* mutations are clearly able to upregulate *CAB* gene expression in the absence of light, implying that the effects of the *hp* mutations on gene expression are rather complex. But clearly, the effects on gene expression, the elevated anthocyanin biosynthesis, and the chloroplast development observed in the roots of these mutants infers that both the *HP1* and *HP2* gene products act as repressors of light signalling and that their loss of function results in the inability to repress a wide range of photoresponses.

Recent studies have demonstrated that *hp2* represents a mutation in the gene encoding the tomato homologue of the *Arabidopsis* nuclear protein DET1, a negative regulator of photomorphogenesis in *Arabidopsis* (Mustilli et al., 1999), and that *hp1* represents a mutation in the tomato homologue of the human and *Arabidopsis* gene encoding the UV-Damaged DNA-Binding protein 1 (DDB1) (Liu et al., 2004). Interestingly, these proteins have been found to interact in the same complex, which has been proposed to regulate photomorphogenic genes through chromatin modifications by interacting with histone H2B tails (Benvenuto et al., 2002; Schroeder et al., 2002). It is therefore not surprising that *hp1* and *hp2* mutants should display such similar phenotypes. It will therefore be of interest to determine whether the differences reported here are due to subtleties in the specific functions of the DET1 and DDB1 subunits within the complex, or whether they are a result of differing relative strengths of the *hp* mutant alleles studied.

Like the *hp* mutants, *atv* mutants also displayed light hyperresponsive phenotypes in both white light and natural light conditions, but only in terms of anthocyanin content and not hypocotyl or stem length inhibition (Fig. 2.5 and 2.6). These results confirm previous observations, although Kerckhoffs et al. (1997[b]) also reported a reduction in hypocotyl length in *atv* mutants. The difference of the current results with this previous report may be a consequence of the differing genetic backgrounds, and it should be noted that the genetic material used here had been backcrossed two times more than was done in the previous study. Furthermore, these results also correlated with the gene expression analysis, in which *CHS* gene expression levels were higher in *atv* mutants than in wild-type seedlings in white light (Fig. 2.25). *CAB* gene expression was nonetheless unaffected in *atv* mutant seedlings, both in the dark and in response to light.

In the current study, microscopic observations of anthocyanin distributions and of plastid development were also performed, which further extended previous observations about the *atv* mutant. Although the increases in anthocyanin content were generally not as high as observed in the *hp* mutants, the cellular distribution of anthocyanin was similar, in that most of the anthocyanin was found in the sub-epidermal layer of the hypocotyls and in the leaf sclerenchyme cells and trichomes (Fig. 2.18). However, anthocyanin biosynthesis was not deregulated in *atv* mutant roots, as was found in the *hp* mutants. In white light conditions, the *atv* mutants nonetheless displayed green roots (indicative of chloroplast development) much the same as the *hp* mutants (Fig. 2.16). Both confocal and electron microscopic techniques revealed that roots of *atv* mutant seedlings contained partially developed chloroplasts, unlike wild-type seedlings (Figs. 2.16 and 2.19). Furthermore, cotyledons of dark-grown *atv* mutant seedlings contained partially developed plastids, albeit not to the same extent as was seen in the *hp* mutants (Fig. 2.19). In *Arabidopsis*, mutations in each of the 11 *cop/det/fus* loci result in the absence of etioplasts and in partial chloroplast development in cotyledons of seedlings grown in complete darkness (Castle and Meinke 1994; Chory and Peto 1990 and Kwok et al., 1996). The same wild-type loci also suppress chloroplast development in the roots of light grown seedlings, because mutant roots develop chloroplasts and become green. It is interesting to find similar phenotypes in the tomato *atv* mutant, inferring the functional importance of the wild-type gene product in light signalling, even though it remains unidentified. Both the recessive nature of all these mutations in *Arabidopsis* and tomato, as well as their pleiotropic phenotypes, could suggest that chloroplast development is the default route of plastid development, and that the etiolation pathway is an evolutionarily recent adaptation pioneered by the angiosperms.

In the continuous broadband experiments the *atv* mutant seedlings showed only slight hyperresponsiveness to the light conditions used and only in far/red light conditions did their hypocotyls show a slightly more pronounced increase in anthocyanin content (Fig. 2.23). Unlike the *hp* mutants, they did not die in far red light. Similar results were also observed by Kerckhoffs et al. 1997[c], in which they found that the *atv* mutants showed the strongest amplification of the far-red HIR component. However, the general lack of hyperresponsiveness to the specific light wavelengths tested here might suggest that phyB and cryptochrome signalling do not depend strongly on the *ATV* gene product. It might therefore be worthwhile to test the responsiveness of *atv* mutant seedlings to UV light, since no UV

light responses have been examined to date in *atv* mutants. In particular, low levels of UV-B are an integral component of incident sunlight and constitute an important environmental factor regulating plant growth and development (Rozema et al., 1997). Only a relatively small fraction of plant photomorphogenesis research has been performed on UV-B-induced photomorphogenesis and the genetic approaches that have been so successful in unravelling the mechanisms of phytochrome and cryptochrome action have not been widely applied to the study of UV-B responses. Recently Boccalandro et al. (2001) reported that UV-B in low doses could mimic the effects of FR and blue light on cotyledon opening and hypocotyls. However, they also concluded that UV-B is not perceived by phytochromes or cryl, which might suggest that a separate photoreceptor system is activated. In this regard, very recently Ulm et al. (2005) described an extensive assessment of the *Arabidopsis* UV transcriptome at the genome-wide level and linked the key photomorphogenesis transcription factor HY5 to responses to the UV-B region of the light spectrum. They also concluded that UV-B mediated transcriptional activation of HY5 is independent of phyA and phyB, suggesting an alternative input pathway for its transcriptional regulation. The general lack of hyperresponsiveness of *atv* mutants to the light conditions tested here might support the idea that they are defective in either a UV-B receptor or downstream signal transduction component.

Another interesting mutant in this study was *Anthocyanin fruit* (*Af*). A single paragraph note originally reported the presence of the dominant gene *Af*, derived from a *L. esculentum* X *L. chilense* cross (Giorgiev 1972). Giorgiev (1972) reported that the purple colour was caused by anthocyanin, although scant evidence was presented to confirm that the pigment was indeed anthocyanin. This was confirmed in the present study. In addition to the higher anthocyanin accumulation found in the fruits of *Af* mutants, white light grown seedlings also showed increased anthocyanin pigmentation in the same cell types as in *hp* and *atv* mutant seedlings, albeit to a lesser extent (Fig. 2.6 and 2.10.). Exaggerated anthocyanin biosynthesis in response to both white light and natural light was particularly apparent in hypocotyls (Fig. 2.6 and 2.10), and hypocotyl length of *Af* mutant seedlings was generally also slightly shorter than wild-type seedlings (Fig. 2.5). These results were however not conclusive because the genetic background of *Af* is not known. An *Af* mutant that had been backcrossed twice to Money Maker nonetheless showed similar, albeit weaker, responses. In spite of these exaggerated photoresponses, the roots of *Af* mutant seedlings did not accumulate anthocyanin nor chloroplasts. In the continuous broadband experiments the *Af* mutant seedlings showed

slightly exaggerated responses to both blue and red light although not to far red light (Figs 2.21, 2.22 and 2.23), indicating that the *Af* mutants might be overresponsive to phyB and cryptochrome-controlled responses. In the gene expression studies *CHS1* expression appeared to be slightly higher in *Af* mutant seedlings than wild-type seedlings when grown in white light, as was *CAB* gene expression (Fig. 2.25).

In conclusion, the results presented in this study have revealed further insights about *hp1* and *hp2* mutant phenotypes, some of which were not previously described. Furthermore, characterization of the *atv* and *Af* mutants has revealed novel characteristics and suggest that these mutants do not have obvious counterparts in *Arabidopsis*. Efforts to clone these genes therefore appear to be well founded, and their eventual identification may provide novel information about the regulation of photomorphogenesis in tomato.

2.6 Materials and methods

2.6.1 Plant Material

Name (abbreviation)	Genetic Background	TGRC number	Seed batch	References
GT (GT)	GT	LA4011	**N990375**	
high pigment-1 ($hp\text{-}1^{w}$)	GT	LA4012	N990381	Reynard, 1956.
Money Maker (MM)	MM	LA2706	**N990376**	
high pigment-2 ($hp\text{-}2^{j}$)	MM	LA4014	N990386	Soressi, 1975.
Anthocyanin fruit/MM (Af/MM)	MM	---------	N000030	Van Tuinen et al., 1997.
Anthocyanin fruit (Af)	Unknown	LA1996	N990031	Georgive, 1972.
Walter (WA)	WA	LA3065	**N000242b**	
dark green (dg)	WA	LA3011	N000286a	Konsler 1973.
Ailsa Craig (AC)	AC	LA2838	**N990374**	
Punctate (Pn)	AC	LA3089	N000274	Rick, 1966.
atroviolacea (atv)	AC	LA0797	N000264	Rick, 1961.
anthocyanin looser	AC	LA3099	N000281	Butler, 1973.

Table 2.1. Several light hyperresponsive mutants and their respective wild types of *Solanum lycopersicon*. The details of mutant plants used in this Chapter are listed here. The TGRC number refers to the stocks as recorded in the Tomato Genetic Resource Center database (http://tgrc.ucdavis.edu). The second number refers to the batch of seeds in the tomato seed bank of SZN. Wild types genotypes are indicated in bold, above each of the respective mutants in that genetic background.

2.6.2 White Light Experiments

Seeds were surface sterilized with a 2% (v/v) solution of commercial bleach for 30 minutes and then washed 3 times for 10 minutes with sterile water. Sixteen seeds of each genotype were placed in magenta boxes containing 200 ml of MS-medium with 0.25 % (w/v) phytagel (Sigma). Four replicates of each genotype were sown. The magenta boxes were placed in a carton box under a black velvet cloth in a dark room at 25°C. Germination was checked every 12 h under green safe light from 48 h after sowing. When a maximum number of seeds had germinated during the same 12 h period, the magenta boxes were transferred to white light 24 hours later. Seedlings were grown for 14 days in a 16 h white light (photosynthetically active radiation [PAR, 400-700 nm], 78 μmol m^{-2} s^{-1}; Philips TL36W/84)/ 8 h dark regime at 25 °C, after which samples were taken. Each sample contained material from five seedlings, which had germinated at the same time.

Hypocotyl length and plant height were measured. Subsequently seedlings were divided in hypocotyl, root and cotyledon pairs. Hypocotyls were weighed and anthocyanin was extracted (see below). One cotyledon of each pair was used for anthocyanin, the other for chlorophyll extraction. Roots were weighed and chlorophyll was extracted. All samples consisted of five seedlings.

2.6.3 Anthocyanin Extraction

For the determination of anthocyanin, samples of five hypocotyls or cotyledons were extracted with 1.2 mL of acidified (1% (w/v) HCl) methanol for 24 h in the dark with shaking. A Folch partitioning (Folch et al., 1957) was performed by adding 0.9 mL of H,O and 2.4 mL of chloroform to the extracts and centrifuging it for 30 min at 3600 rpm. The O.D of the top phase was determined with a Beckman DU-64 spectrophotometer at A_{535}.

2.6.4 *Chlorophyll Pigment Analysis*

For chlorophyll extraction, samples of 10 cotyledons were weighed, placed in glass tubes, immersed in a 100 times excess volume of N, N-dimethylformamide (w/v) (Moran, 1982), and incubated in the dark for 24 h. The O.D at A_{647} and A_{664} of the extracts were measured,

and Chl content was calculated on a fresh weight basis using the equations published by Inskeep and Bloom (1985).

2.6.5 Light Microscopy

To observe anthocyanin and chloroplast localization, hand-cut sections were taken from the hypocotyls and from the roots respectively. These sections were examined under the light microscope (Zeiss, Axiolab HBO 50). To show the presence of chloroplasts in the roots it was more convenient to use whole roots, which were taken and observed with a confocal laser-scanning microscope (Zeiss, LSM410, MS scientific, Karlsgartenstrasse 16. 12049 Berlin-Germany).

2.6.6 Electron Microscopy

To examine the ultrastructure of the chloroplasts, cotyledons and roots (only from genotypes with chloroplasts in the roots) and their wild types were fixed and embedded. Seedlings and cotyledons were selected under safelight. Cotyledons were cut and fixed with 2% (w/v) glutaraldehyde 2% (w/v) paraformaldehyde in 0.05 M cacodylatebuffer. Samples were put under vacuum for 10 min and fixed with 2% (w/v) glutaraldehyde 2% (w/v) paraformaldehyde in 0.05 M cacodylatebuffer for 4 hrs with shaking at room temperature. Samples were washed 3 times 5 min each with 0.05 cacodylatebuffer. Postfixation was done with 2% (v/v) OsO_4 for 2 hrs at room temperature. Samples were washed 4 times 5 min each with 0.05 M cacodylatebuffer. Samples were contrasted with 1% (w/v) uranylacetate overnight (16 hrs) at 4 ^{0}C. Samples were then washed 3 times 5 min each with mQ H_2O and dehydrated with acetone series, 15 min each step: 10%, 30%, 50%, 70%, 80%, 90%, 100%, 100% (v/v). Dehydrated samples were impregnated series of increased concentration of ERL (= Spurr's resin). 25% (v/v) ERL in ethanol for 3 hrs while shaking at room temperature 50% (v/v) ERL in ethanol for 3 hrs while shaking at room temperature 75% (v/v) ERL in ethanol for 16 hrs at 4 °C. 100% (v/v) ERL for 3 hrs while shaking 100% (v/v) ERL for 4 hrs while shaking. Embedded samples in a flat embedding mould and polymerised in the oven at 70°C for 10 hrs. Thin sections were stained with 1% (v/v) uranyl acetate and 3% (v/v) lead citrate and examined by transmission electron microscopy (Philips, model 400, Philips Medical Systems, Castlefiled Road, Reigate, Surrey, RH2 0FY). This work has been done in

collaboration with Christy Efde and the Electron microscopic facility at the Stazione Zoologica, Naples.

2.6.7 Natural White Light Experiments

Tomato plants were grown for one month in a greenhouse in Pagani near Naples under natural light conditions. The first white light experiment was carried out in November and the second white light experiment in January-February. The minimum temperature during the night was 10°C. Germination was checked every two days.

One month after germination, hypocotyl length and plant height were measured. Samples of five hypocotyls were weighed and anthocyanin was extracted from the first white light experiment whereas from the second white light experiment single hypocotyl samples were used. A minimum of four samples from each genotype was taken for white light experiment 3 and twelve samples for white light experiment 5. For both experiments, five young leaves from five plants were sampled, weighed and anthocyanin was extracted (see below). Five more young leaves of the same five individual plants were sampled, weighed and chlorophyll was extracted. A minimum of four replicates were taken from both experiments.

Hand-cut sections were taken from the lowest part of the hypocotyl (0.5-1.0 cm above the soil) and from the middle part of young leaves and examined by light microscopy.

2.6.8 Dark Experiment

Seeds were surface sterilized as described above and sown on MS-medium with 0.25% (w/v) phytagel (sixteen seeds per magenta box, three magentas per genotype). Two magentas containing seeds of each genotype were wrapped in aluminium foil and placed in carton boxes. They were then placed in a dark room (25°C) and covered with a black velvet cloth. To keep track of germination under the specified conditions, magenta boxes that were not wrapped in aluminium foil were checked for germination every 12 hrs under dim green safe light. Five days after germination of seeds in the magenta boxes where germination had been checked, cotyledons from the four longest seedlings grown in absolute dark (*i.e.* in wrapped magenta's) were fixed in total darkness and processed as described above.

2.6.9 Continuous Broad-band Light Experiments

Non-pre-treated Money Maker (WT), GT (WT), Ailsa Craig (WT), Walter (WT), *atv*, *Af*, *hp1*^{w}, *hp2*^{j}, and *dg* seeds were directly sown in plastic trays filled with potting compost and incubated in the dark for 3 days at 25 ^{0}C. Irradiation with continuous B, R and FR (3-µmol m^{-2} s^{-1}) was begun just before the seedlings emerged through the soil surface. The length of 10 hypocotyls was measured after 7 days. These hypocotyls were also used for anthocyanin extraction. The cotyledons of the same seedlings were divided into 2 parts and one part was kept for anthocyanin extraction and the other part was kept for chlorophyll extraction. In addition similar extractions and measurements were done with the seedlings grown in absolute darkness at the same time.

2.6.10 Molecular Analysis

For isolation of RNA, 25 cotyledons with apical hooks attached were collected from the seedlings grown for 5 days in the dark and transferred to white light. After transfer to white light, samples were collected at 0, 3, 6, 9 and 12-hour intervals. These samples were ground to a fine powder in liquid nitrogen and subsequently extracted using the hot phenol method (DellaPenna *et al.*, 1986). To the grounded powder 5mL of extraction buffer (100mM tris pH8.0, 10mM EDTA and 1%SDS) and 5mL of Phenol mix, which was preheated to 80 ^{0}C, was added and vortexed vigorously. The samples were spun at 5000 rpm for 10min and the aqaeous phase was carefully removed into a new falcon tube. The samples were extracted once with Chloroform isoamyl alcohol (24:1), an eqval volume of 4M lithium chloride was added and the samples were kept at -80 ^{0}C for 2 hours. The RNA was washed with 70% (v/v) EtOH and suspended in 200-400 µl of DEPC water. 10 µg of RNA was loaded onto formaldehyde gels and blotted overnight onto Hybond N+ membranes (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire HP7 9NA, England) using 10X SSC. Hybridization was carried out overnight using an α-^{32}P-labelled tomato *CHS1* probe (O'Neill et al., 1990) with Church and Gilbert buffer. The washes were performed in 0.1% (w/v) SDS and 1X SSC twice for 5 minutes each. Blots were stripped using hot SDS (1%) and re-probed with an α-^{32}P-labelled tomato *CAB6* probe (Piechulla et al., 1991). The blots were exposed using Kodak autoradiography film for 5 hours and developed.

CHAPTER 3

Manipulation of *DET1* Expression in Tomato Results in Photomorphogenic Phenotypes Caused by Post-Transcriptional Gene Silencing

3.1 Summary

The tomato *HIGH PIGMENT 2* gene encodes a homologue of the *Arabidopsis* nuclear protein DE-ETIOLATED 1 (DET1). From genetic analyses it has been proposed that DET1 is a negative regulator of light signal transduction, and recent results indicate that it may control light-regulated gene expression at the level of chromatin remodelling. To gain further understanding about the function of DET1 during plant development, a range of overexpression constructs were generated and introduced into tomato. Unexpectedly, phenotypes characteristic of DET1 inactivation, i.e., hyper-responsiveness to light were observed. Molecular analysis indicated in all cases that these phenotypes were a result of suppression of endogenous *DET1* expression, due to post-transcriptional gene silencing. *DET1* silencing was often lethal when it occurred at relatively early stages of plant development, whereas light hyper-responsive phenotypes were obtained when silencing occurred later on. The appearance of phenotypes correlated with the generation of siRNAs but not DNA hypermethylation, and was most efficient when using constructs with mutations in the *DET1* coding sequence or with constructs containing only the 3'-terminal portion of the gene. These results indicate an important function for DET1 throughout plant development and demonstrate that silencing of *DET1* in fruits results in increased carotenoids, which may have biotechnological potential.

3.2 Introduction

An important class of Arabidopsis mutants display light responses in darkness and are known as *constitutive photomorphogenic* (*cop*) or *de-etiolated* (*det*) mutants (Schafer and Bowler, 2002). A total of 11 such loci have now been described. Identification of the genetic lesions in these mutants has led to the elucidation of important mechanisms controlling photomorphogenic responses, such as polyubiquitin- mediated proteolysis of positively acting signalling intermediates, e.g., the transcription factor HY5 (Serino and Deng, 2003). Tomato (*Solanam lycopersicon*) has become a model system complementary to Arabidopsis for studying light responses, allowing general conclusions to be drawn about the roles played by individual photoreceptors in higher plants (Kendrick et al., 1997). Analysis of photoreceptor function in tomato also allows study of their roles during fruit development, which is more difficult in *Arabidopsis*.

It has been known for 50 years that phytochromes can regulate the fruit ripening process (Piringer and Heinze, 1954). More recent studies have confirmed that red and far-red light can penetrate the epidermis and pericarp of both immature and mature tomato fruits, and that phytochromes are indeed present in fruit tissues (Alba et al., 2000). Other studies have shown that cryptochromes also play an important role in fruit ripening (Weller et al., 2001).

In tomato, no constitutive photomorphogenic mutants have ever been found. However, mutants with an exaggerated light responsiveness have been identified such as the *high pigment* mutants *hp1* and *hp2* (Kendrick et al., 1997). Light-grown *hp* mutants display high levels of anthocyanins, are shorter and darker than wild-type plants and have dark green immature fruits, so it is likely that they are mutated in genes encoding important negative regulators of photoreceptor- linked signal transduction pathways. Cloning of the *HP2* gene indeed revealed that it encodes the tomato homologue of *Arabidopsis* DET1 (Mustilli et al., 1999). This was nonetheless surprising, given that *Arabidopsis det1* mutants display constitutive photomorphogenesis (Chory et al., 1989), whereas *hp2* mutants do not (Mustilli et al., 1999). However, it is not known whether *hp2* mutant alleles represent null alleles, and consequently the effect of *DET1* inactivation in tomato is not known. DET1 is a nuclear protein (Mustilli et al., 1999; Pepper et al., 1994), and recent findings suggest that the functional form of DET1 is within an approximately 350 kDa complex that also contains the

plant homologue of UV-damaged DNA binding protein 1 (DDB1) (Schroeder et al., 2002). Interestingly, the *hp1* mutant is now known to be mutated in *DDB1* (Lieberman et al., 2004; Liu et al., 2004). Furthermore, Benvenuto et al. (2002) have shown that DET1 binds to hypo-acetylated amino-terminal tails of the core histone H2B and have proposed that DET1 may be involved in chromatin remodelling around photoregulated genes (Schafer and Bowler, 2002). For example, DET1 may limit access of positive regulatory factors to the promoters of light responsive genes. This Chapter summarizes the analysis of phenotypes of the transgenic tomato plants in which *DET1* gene expression has been modulated. A number of independent transgenic lines have been generated containing different *DET1* constructs and the range of phenotypes observed is shown. Surprisingly, in all cases phenotypes were a consequence of the induction of post-transcriptional gene silencing (PTGS) of the *DET1* gene. The data presented here support the notion that DET1 is an important regulator of photomorphogenesis that plays a role during the entire life cycle of the plant.

3.3 Results

3.3.1 Transgenic Plants Containing Tomato DET1 (TDET1) Constructs Show a Range of Light Hyper-Responsive Phenotypes

To investigate the function and importance of DET1 in tomato, several transgenic lines were generated containing different forms of the *TDET1* gene. Table 3.1 summarizes these constructs, the number of lines generated and the number of lines showing clear phenotypes. A total of 11 constructs were utilized, which contained three different promoters (CaMV 35S and enhanced 35S (e35S) promoters for constitutive transgene expression (Benfey and Chua, 1990) and the E8 promoter for fruit specific expression (Deikman et al., 1992), with different *TDET1* transgene constructs (including sense, antisense, *hp2* and *hp2j* mutant alleles, 5'-terminal and 3'-terminal), and two different genotypes (Money Maker and T56) (see Experimental procedures). The constructs were generated by Anna Chiara Mustilli at the host laboratory and by Seminis Vegetable Seeds (Woodland, CA-USA). Full-length *TDET1* expressed from either the 35S or the e35S promoter was expected to generate light-insensitive phenotypes, whereas 5'-terminal and 3'-terminal constructs were made in an attempt to identify dominant negative constructs. Antisense constructs were expected to repress *TDET1*

gene expression and consequently to generate light hyper-responsive phenotypes. However, when phenotypes were visible they were invariably characteristic of exaggerated light sensitivity, i.e., shorter bushy plants and dark green immature fruits, reminiscent of high pigment mutants (Kendrick et al., 1997) (Figure 3.1).

From the data in Table 3.1, several observations can be made: (i) All sense-oriented *TDET1* constructs produced hypersensitive phenotypes in at least one genetic background when expressed from a 35S-based construct, (ii) Hypersensitive phenotypes were observed in both Money Maker and T56 genotypes at similar frequencies, (iii) Expression of transgenes using an e35S promoter was slightly more effective in generating hypersensitive phenotypes than was the 35S promoter, (iv) Plants containing antisense *TDET1* constructs displayed no obvious phenotypes, (v) Constructs containing the fruit-specific E8 promoter produced no phenotypes.

Beyond this simplified summary, notable differences were observed, including the severity of phenotypes (i.e., more dwarf statures and darker green immature fruits) generated by different constructs (Table 3.1). In particular, transgenes containing the *hp2* and *hp2^j TDET1* mutant alleles produced more dramatic phenotypes compared with the *TDET1* wild-type transgene, and the truncated 3'-terminal construct tended to produce plants with more severe phenotypes than the full length or truncated 5'-terminal constructs. These phenotypes are illustrated in Figure 3.1 and are more thoroughly described below.

Construct	Genotype	No. lines	No. lines with *hp*-like phenotypes	Severity of Phenotypes[2]
35S::TDET1	MM	5	0	-
35S::TDET1-5'	MM	3	2	+
35S::TDET1-3'	MM	2	0	-
35S::TDET1-AS	MM	7	0	-
35S::*hp2*	MM	3	3	+++
	T56	5	3	+++
35S::*hp2*^{i}	MM	5	0	-
	T56	13	5	+++
e35S::TDET1	T56	45	14	+
e35S::TDET1-5'	T56	15	6	++
e35S::TDET1-3'	T56	8	4	+++
E8::TDET1	MM	17	0	-
E8::TDET1-AS	MM	2	0	-
	T56	19	0	-

[2]+ and ++ denote severity of blotchy phenotypes, +++ denotes hp phenotypes.

Table 3.1 Details of constructs used and number of lines generated.

3.3.2 Range of Phenotypes in Transgenic Plants

Figure 3.1 shows the range of light hyper-responsive phenotypes observed in transgenic plants containing different *TDET1* constructs. In many cases fruit phenotypes ranged from normal wild type to intermediate blotchy (normal fruits with dark green patches) to uniform dark green *high pigment* (*hp*)-like phenotypes (Figure 3.1a–c). As mentioned earlier the full-length *TDET1* construct produced only the weak blotchy phenotype, whereas the truncated and mutant constructs often generated the more severe *hp* phenotypes. Similarly, whole plant phenotypes varied from wild type to intermediate dark green to very dark green bushy plants (Figure 3.1d–f).

Both the 3'-terminal and the *hp2* and *hp2^j* mutant allele constructs produced the most severe whole plant phenotypes, whereas the 5'-terminal construct only gave the intermediate dark green phenotype. In most cases the phenotypes were not visible in seedlings but appeared during the later development of the plants, after at least one month. In some cases, the plants produced wild-type fruits and only subsequently did blotchy and *hp* phenotypes appear. In other cases the plants generated only blotchy or *hp* fruits. In a few cases, most notably with the 3'-terminal and mutant allele constructs the phenotypes were more severe and appeared earliest during plant development compared with plants containing the other constructs. These plants were dark green and often died before producing fruits (Figure 3.1g). Phenotypes caused by the 3'-terminal construct were slightly different than with the other constructs. These plants were severely dwarfed and in the apex of the plant the leaves were arranged much closer together than normal and became progressively yellowish rather than dark green (Figure 3.1h). Finally, blotchy phenotypes varied between the Money Maker and T56 genotypes. In Money Maker the fruits showed dark green blotches (Figure 3.1i), whereas in T56 the dark green patches appeared as more precisely defined sectors (Figure 3.1j).

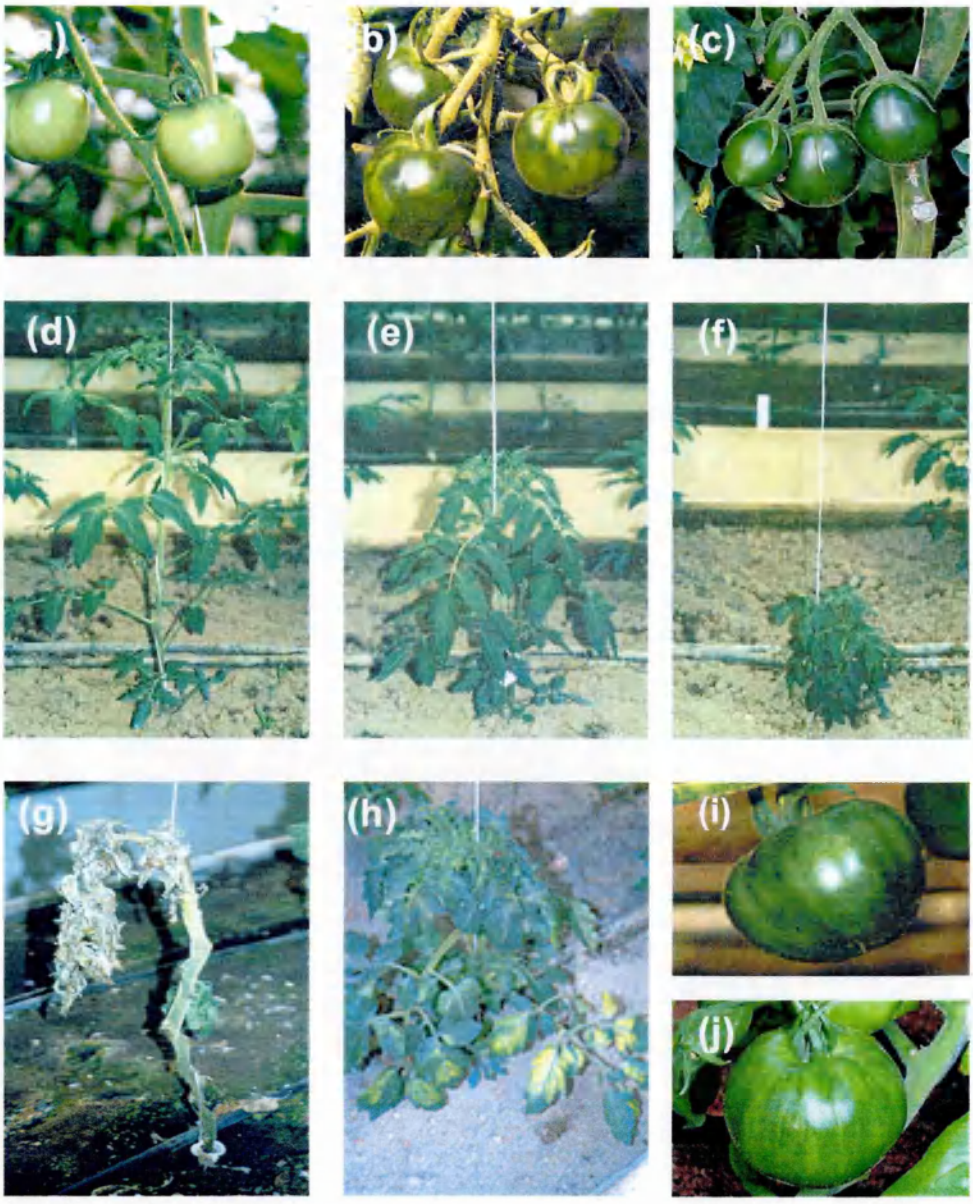


Figure 3.1. Phenotypes of transgenic plants containing different *TDET1* constructs. In all cases [except in (h) and (j)], photographs were taken from MM plants containing the 35S::*hp2* construct. Identical phenotypes were also observed with other constructs (see Table 3.1), albeit with different severity (see text). (a)Wild-type phenotype fruits. (b) Blotchy fruits. (c) *hp*-like dark green immature fruits. (d) Wild-type plant phenotype. (e) Intermediate light hyper-responsive plant phenotype. (f) Severe light hyper-responsive young plant phenotype. g) Severe light hyper-responsive old plant phenotype. (h) Phenotype of MM genotype transformed with 35S::*TDET1*-3 construct. (i) Blotchy fruit phenotype in MM genotype. (j) Blotchy fruit phenotype in T56 genotype.

3.3.3 Fruits from Transgenic Plants Produce High Levels of Pigments

Pigment analysis of immature fruits showing phenotypes confirmed that they contain high levels of chlorophyll (Figure 3.2). There was a clear difference in pigment content between wild type, blotchy and dark green fruits, and these latter fruits contained more than five times the chlorophyll levels of wild-type fruits and four times more than *hp2* mutant fruits (Figure 3.2). The intermediate blotchy fruits contained approximately three times more chlorophyll than wild-type fruits. This was further confirmed by Northern blot analysis on these fruits, which showed upregulation of *CAB* gene expression in *hp*-phenotype fruits from transgenic plants (see Figure 3.3c).

Levels of lycopene and β-carotene in mature red ripe fruits were examined (Table 3.2). Lycopene levels were increased more than twofold in *hp* phenotype fruits compared with wild-type fruits, and β-carotene levels were increased fivefold. Table 3.2 also shows that the brix values and sugar content of the transgenic fruits. Furthermore, the total yield of fruits from plants displaying *hp*-like phenotypes was significantly reduced, which is characteristic of *hp2* mutants (Mustilli et al., 1999). (This biochemical analysis was done in collaboration with Cirio Pvt. Caserta-Italy).

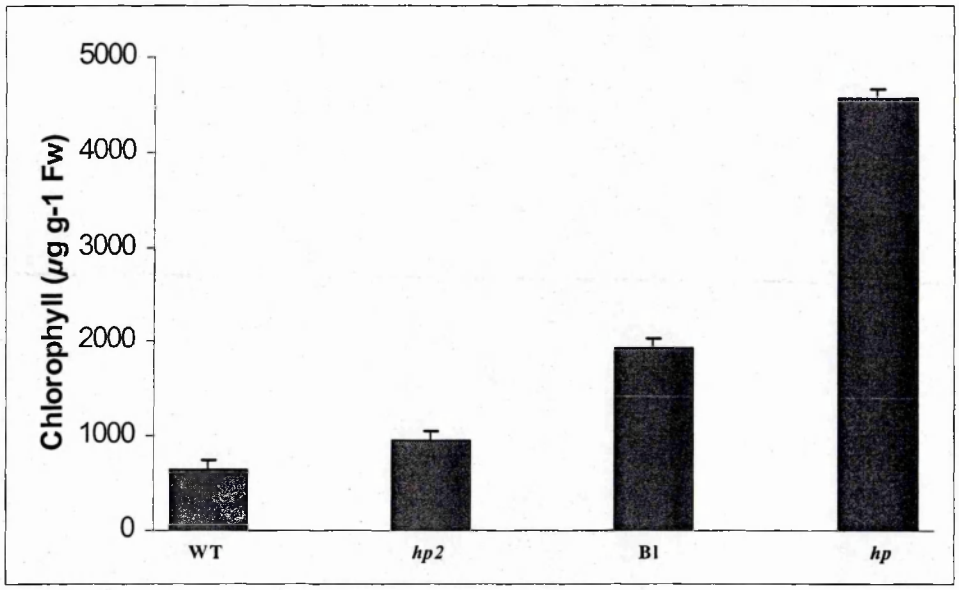


Figure 3.2. Chlorophyll analysis of pericarp from blotchy and *hp* phenotype fruits from MM plants transformed with *35S*::*hp2*. Fruits from MM (WT) and *hp*2 mutants (MM background) were used for comparison. Wild-type (WT), Blotchy (Bl) and *high pigment* (*hp*) fruit phenotypes. Green immature fruits were harvested from five plants per genotype. Standard error bars are shown.

Plant material	Fruit Yield per plant (g)	Brix ($g\ 100g^{-1}$)	Sugars ($g\ 100g^{-1}$)	Lycopene (p.p.m.)	β-Carotene (p.p.m.)	Vitamin A ($IU\ g^{-1}$)[b]
Wild type T56	224 ± 9	4.73 ± 0.06	2.58 ± 0.08	85.7 ± 4.3	3.03 ± 0.41	5.04 ± 0.68
Transgenic T56 with *hp* phenotype	151 ± 29	4.60 ± 0.08	2.30 ± 0.25	186 ± 28.4	15.6 ± 5.69	26.03 ± 9.49

Table 3.2 Pigment analysis of fruits from transgenic lines with *hp* phenotypes[a]

[a]All red ripe fruits were harvested from individual T2 generation plants, weighed, and analyzed as in Materials and Methods. Wild-type material was derived from non-transformed T56 genotype (six plants), whereas plants with hp phenotypes were T2 generation T56 plants containing either the *35S*::*hp2*[j] or *e35S*::*TDET1*-3' constructs (five plants). Plants were grown in Woodland, CA, during the spring of 2002. Standard errors are indicated. [b]International units of vitamin A per gram.

3.3.4 Molecular Analysis of Transgenic Plants

Southern blot analyses were used to confirm the presence of the transgenes (Figure 3.3a) and lines containing single copy inserts were chosen for further molecular analysis. RNA from leaves and fruits of transgenic plants displaying either wild-type or light hyper-responsive phenotypes was extracted and *TDET1* mRNA levels were examined by Northern blot hybridization. Endogenous *TDET1* mRNA is undetectable by this method because the gene is expressed at extremely low levels (data not sown). Consequently, any observed expression should be derived from the *TDET1* transgene. Interestingly, severity of the light hyper-responsive phenotypes was negatively correlated with transgene expression levels (Figure 3.3b,c). Because the endogenous *TDET1* gene is normally expressed at only very low levels RT-PCR analysis was performed with specific primers to determine whether *TDET1* expression levels in transgenic material displaying light hyper-responsive phenotypes were below the levels normally found in wild-type plants. This analysis revealed that *TDET1* expression levels were indeed below normal levels and were often completely undetectable (Figure 3.3d). Furthermore, a negative correlation between expression levels and strength of phenotype was again found in this analysis. These observations therefore demonstrate that loss of endogenous *TDET1* gene expression results in severe light hyper-responsive phenotypes, thereby confirming a previous hypothesis that loss-of-function mutations in the *TDET1* gene are responsible for the *hp2* mutation (Mustilli et al., 1999), thus implying the importance of TDET1 for controlling light responses in tomato. Furthermore, these data indicate that the phenotypes observed were probably caused by suppression of endogenous *TDET1* expression rather than by overexpression of the gene. Therefore the underlying mechanism was investigated and these plants were used for further understanding the importance of DET1 in tomato development.

Based upon phenotypic observations of transgenic plants, suppression of *TDET1* expression appeared to occur at a certain moment and then to spread to neighbouring regions, or even throughout the whole plant (see above). *TDET1* expression levels were therefore examined in RNA extracted from different regions of the same plant. One such example is illustrated in Figure 3.4a, in which *TDET1* expression is shown in fruits from a truss, which displayed

phenotypes ranging from wild type to fully *hp*. This analysis revealed that *hp* fruits had extremely low levels of *TDET1* mRNA, that wild-type phenotype fruits had high levels of *TDET1* transgene mRNA, and that blotchy fruits had intermediate levels (Figure 3.4a). Furthermore, in such cases the oldest fruits were visually dark green, and a gradient of phenotypic severity was often observed in fruits at different distances. This, as well as the observed sudden death of healthy plants in some cases (see Figure 3.1g), inferred the systemic spread of a silencing signal from one area of the plant to another. Such a phenomenon has been found to occur during post-transcriptional gene silencing (PTGS) (Vaucheret and Fagard, 2001).

It was curious that no phenotypes characteristic of *DET1* overexpression, i.e. light insensitivity, were ever observed, even with constructs designed to overexpress the full-length wild-type gene. This could be because the constructs were not functional, i.e. incapable of generating a functional protein, or because overexpression was lethal. To test this, the *35S::TDET1* construct was transformed into the *hp2* mutant. The fruit phenotypes of these plants demonstrated that the transgene could indeed complement the phenotypes of *hp2* mutants, confirming that it was functional (Figure 3.4b). Nonetheless, in some cases complementation was only partial because fruits sometimes displayed sectors with normal pigmentation on a dark green background (Figure 3.4b(iii)). These observations were again reminiscent of *TDET1* gene silencing

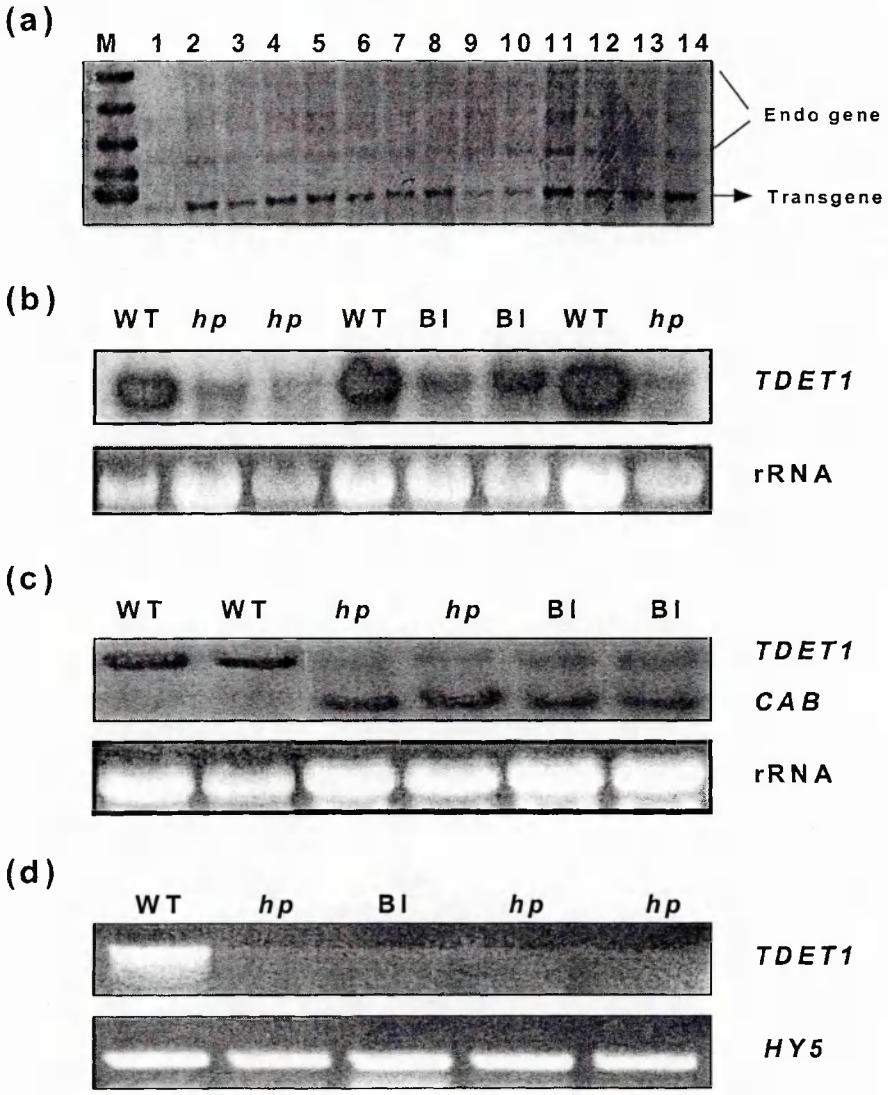


Figure 3.3. Molecular analysis of transgenic plants showing light hyper-responsive phenotypes. (a) Southern blot analysis of transgenic plants contaning the *35S::TDET1* construct. T2 transgenic plants were digested with *Hind*III and probed with *TDET1*. (b) Northern blot analysis of *TDET1* mRNA levels in leaves of different transgenic lines showing light hyper-responsive phenotypes. (c) Northern blot analysis of *TDET1* and *CAB* mRNA levels in fruits of different transgenic lines showing light hyper-responsive phenotypes. (d) RT-PCR analysis of fruits from different transgenic lines showing light hyper-responsive phenotypes. Full-length *TDET1* was synthesized using specific primers and HY5 primers were used as a control for cDNA synthesis and loading. In (b) and (c) 28S rRNA is shown as loading control. MM plants containing the 35S::*hp2* construct were used for all analyses. Abbreviations: WT, Wild-type; Bl, Blotchy and *hp*, *high pigment* (transgenic phenotypes).

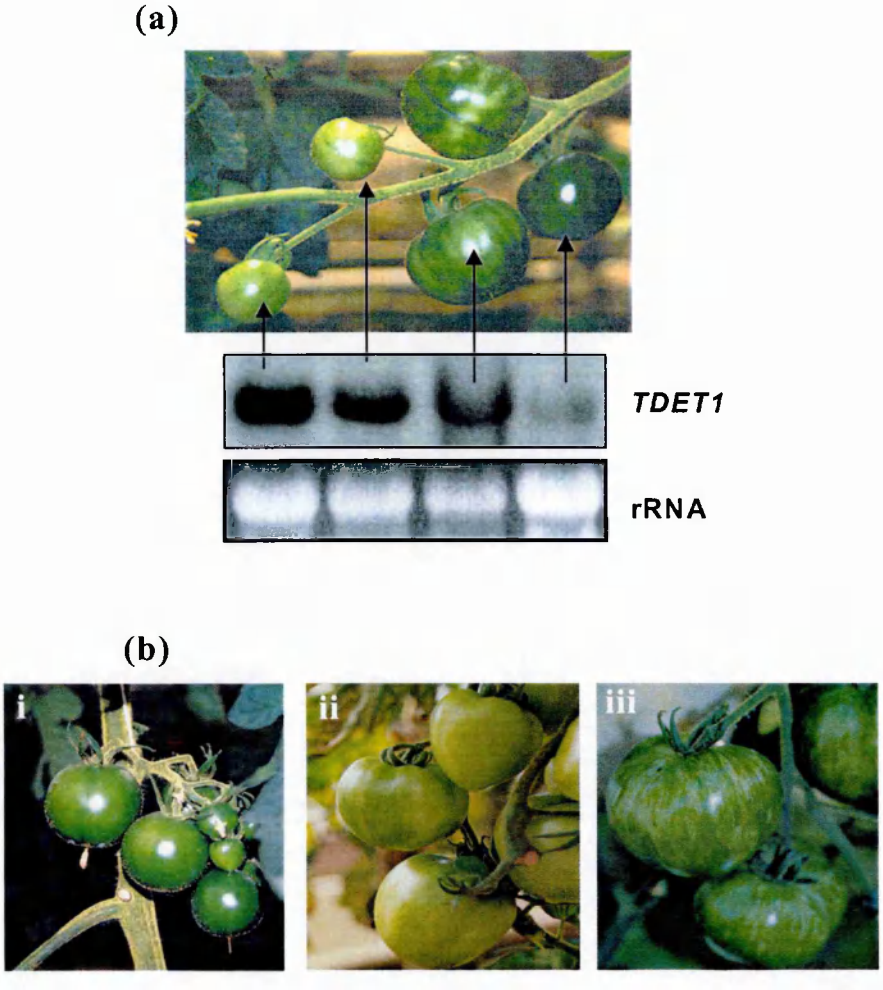


Figure 3.4. Molecular analysis of the transgenic plants. (a) Northern blot analysis of *TDET1* mRNA levels in fruits from the same truss. Severity of light hyper-responsive phenotypes correlated negatively with expression levels of *TDET1*. 28S rRNA is shown as loading control. (b) Complementation of *hp2* mutant phenotype with 35S::*TDET1* construct. (i) *hp2* mutant fruits. (ii) Fruits from an *hp2* mutant plant containing the 35S::*TDET1* construct. (iii) Blotchy fruits from an *hp2* mutant plant containing the 35S::*TDET1* construct, indicative of only partial complementation.

3.3.5 TDET1 Silencing is Inherited Epigenetically

Analysis of MM and T56 transgenic plants from up to five generations showed that phenotypes often reappeared but that they were not inherited normally, e.g., seeds from a plant producing *hp* fruits gave rise to plants with both wild-type and *hp* phenotypes that segregated in a non-Mendelian fashion. For example, out of 10 plants sown in the T2 generation from a WT T1 plant, three produced *hp* fruits, three blotchy and four were phenotypically wild type (Figure 3.5). In the next generation, eight *hp*, one blotchy and one wild-type phenotype plant were obtained from seeds derived from a blotchy phenotype T2 plant. Furthermore, penetration of the *hp* phenotype increased in successive generations and sometimes reached 100% by the fifth generation (data not shown). Following germination, seedlings invariably displayed wild-type phenotypes and the onset of the appearance of light hyper-responsive characteristics occurred during the course of development of the plant, typically after at least 1 month. Because loss of DET1 activity results in easily observable phenotypes in seedlings, e.g., shorter hypocotyls and increased anthocyanin content (Mustilli et al., 1999), these data indicate that the light hyper-responsive phenotype of *TDET1*-suppressed plants is not inherited but that it must be acquired each time by successive generations. The phenomenon therefore has an underlying epigenetic basis.

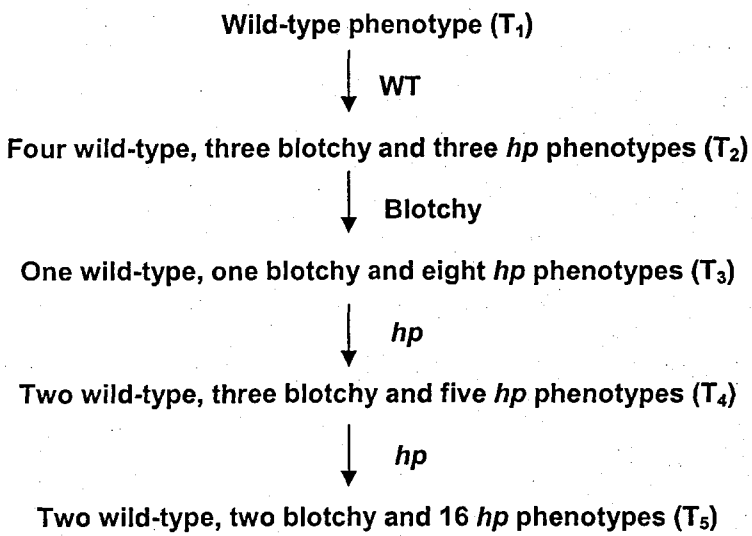


Figure 3.5. Phenotypes of plants in successive generations. The example shown is from an MM transformant containing a single copy of the 35S::*hp2* construct. Phenotypes were scored in five successive generations. All plants were kanamycin-resistant and contained the *TDET1* transgene (verified by PCR).

3.3.6 Light Hyper-Responsive Phenotypes in Transgenic Plants are Caused by PTGS of the TDET1 Gene

Systemic spread of gene silencing and its epigenetic inheritance are characteristic features of PTGS (Vaucheret and Fagard, 2001). A diagnostic molecular characteristic of PTGS is the appearance of short interfering RNAs (siRNAs) of between 21 and 25 nucleotides (Hamilton and Baulcombe, 1999). To detect these low molecular weight RNA from leaves and fruits of transgenic plants was isolated, run on denaturing polyacrylamide gels, blotted onto nylon membranes, and hybridized with an in vitro transcribed *TDET1* antisense probe. Figure 3.6 shows that *TDET1*-derived siRNAs were indeed detected in the plant material displaying *hp* phenotypes. The molecular weight of the siRNAs was 25 nucleotides. Furthermore, the amounts of *TDET1*-derived siRNAs were directly proportional to the severity of the light hyper-responsive phenotypes and in particular were present at the highest levels in plants containing *TDET1-3'* or the *hp2* mutant allele construct (Figure 3.6a). Interestingly, the *TDET1-3'* construct was much more efficient at siRNA generation than the *TDET1-5'* construct (Figure 3.6a), a result which was confirmed by examining eight transgenic plants from several independent lines containing each construct (Figure 3.6b). Conversely, semi-quantitative RT-PCR analysis of *TDET1* mRNA levels showed that material from *TDET1-5'* transgenic plants contained slightly more full-length *TDET1* mRNA than did *TDET1-3'*-containing plants (Figure 3.6c). Levels were nonetheless lower than in plants with wild-type phenotypes. These results therefore demonstrate that the 3'-terminal construct was more efficient in generating siRNAs than was the 5'-terminal construct, which also correlated with the severity of the observed phenotypes (i.e. strong hp phenotypes were never observed in plants containing the *TDET1-5'* constructs). The same result was observed with the *hp2* and *hp2^j* constructs with respect to full-length wild-type *TDET1* constructs (data not shown).

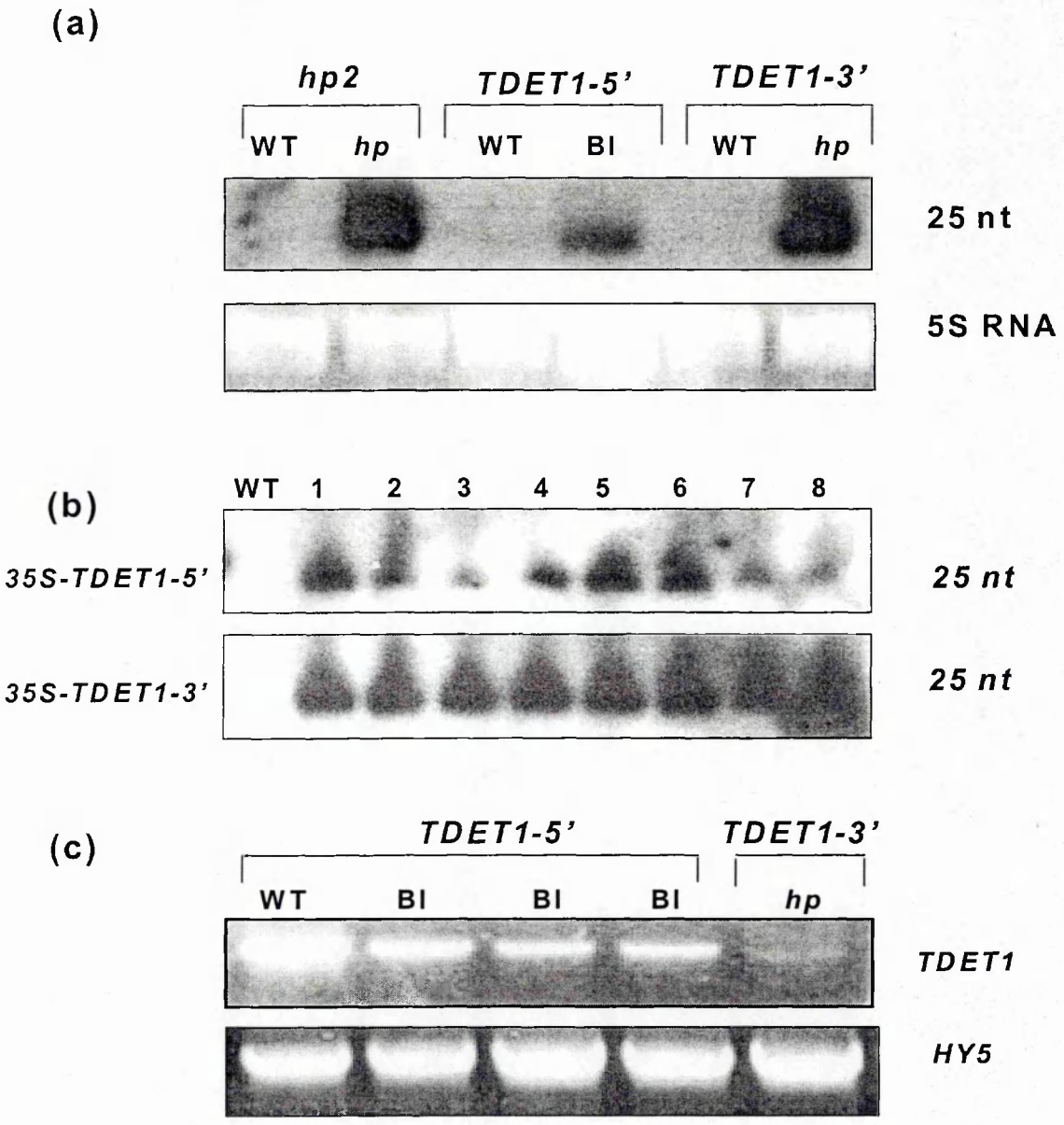


Figure 3.6. Molecular analysis of *TDET1* gene silencing. (a) siRNA analysis of transgenic lines overexpressing three different *TDET1* constructs. In vitro-synthesized *TDET1* was used as probe. 5S rRNA is shown as loading control. (b) siRNA analysis of eight independent transgenic plants containing *TDET1-5'* and *TDET1-3'* terminal constructs. In vitro-synthesized *TDET1* was used as probe. (c) RT-PCR analysis of transgenic lines containing e35S::*TDET1-5'* and e35S:: *TDET1-3'* constructs. Full-length *TDET1* was synthesized using specific *TDET1* primers and *HY5* primers were used as control for cDNA synthesis and loading. 35S::*hp2* plants were MM genotype, whereas the e35S::*TDET1*-3 plants were T56 genotype.

Finally it was tested whether silencing of the *TDET1* gene was also caused by methylation of the *TDET1* coding sequence, which is another characteristic of PTGS (Vaucheret and Fagard, 2001). This was done using a range of methylation sensitive restriction enzymes on genomic DNA extracted from plants containing either *hp2* or *TDET1-3'* constructs. Southern blot analysis revealed no differences between silenced and wild type plants, indicating that neither the *TDET1* endogene nor the transgene were methylated (Figure 3.7a).

As a more efficient method to examine DNA methylation the McrPCR technique was used, which has been developed recently for high-throughput methylation analyses (Lippman et al., 2003). McrBC is an endonuclease, which only cleaves DNA containing methylcytosine residues on one or both strands, so the successful amplification of DNA after digestion with McrBC indicates the lack of methylation. Genomic DNA (2 μg) from both wild-type and silenced plants was digested with McrBC for different times and 50 ng of genomic DNA was then used to amplify the *TDET1* gene. Figure 3.7b shows the successful amplification of the *TDET1* transgene from both wild-type phenotype and silenced plants, indicative of the absence of methylation. Similar analyses were performed using intron-derived primers to amplify the *TDET1* endogene and again no evidence for methylation was found (data not shown). These results suggest that the observed light hyper-responsive phenotypes were caused by PTGS acting at the level of mRNA degradation and siRNA generation rather than through DNA hypermethylation.

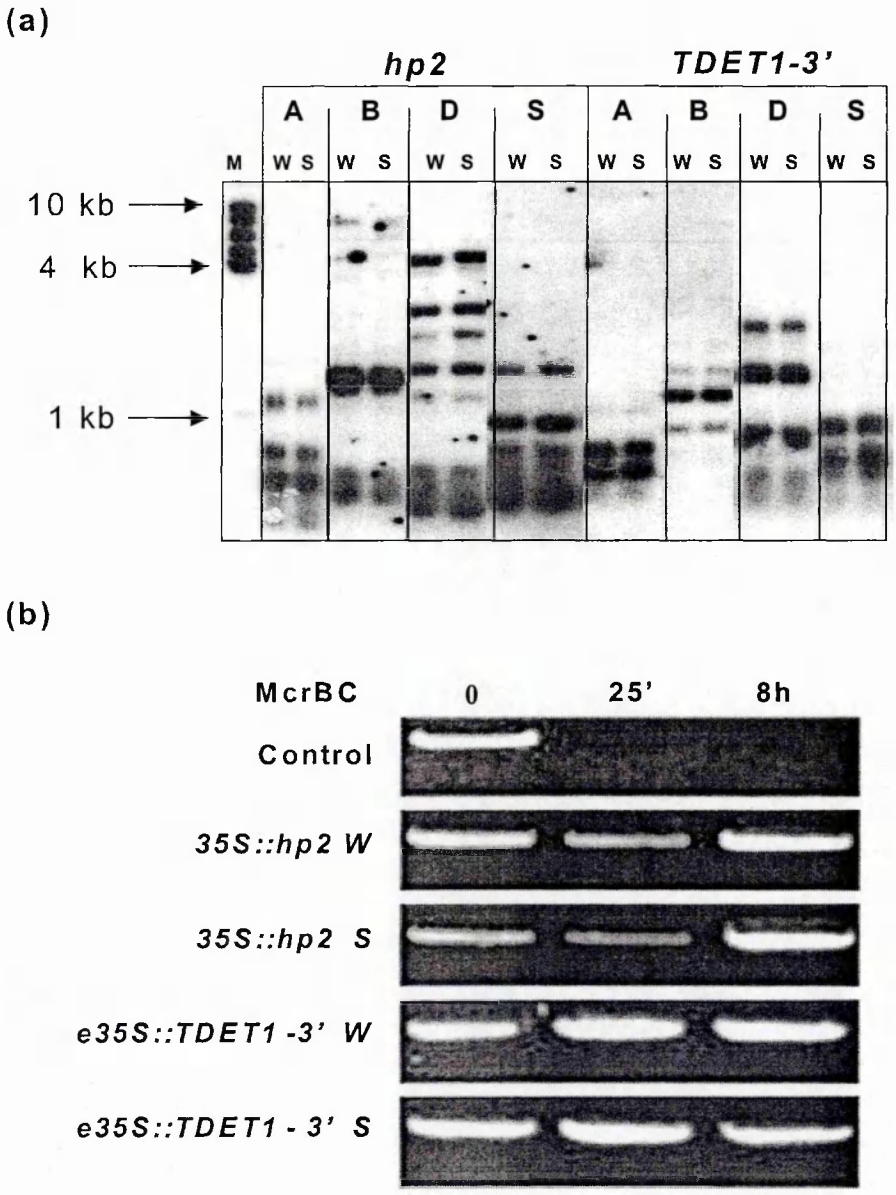


Figure 3.7. Methylation analysis of *TDET1* silenced plants. (a) Methylation analysis of genomic DNA from transgenic plants containing 35S::*hp2* and e35S::*TDET1-3'* constructs. Genomic DNA from wild type (W) and fully silenced (S) plants was digested with *AluI* (A), *BglII/EcoRI* (B), *DdeI* (D) and *Sau3AI* (S). Full-length *TDET1* was used as probe. 1 kb size markers are indicated. (b) McrPCR analysis of genomic DNA from transgenic plants containing *35S::hp2* and *e35S::TDET1-3* constructs. Genomic DNA from wild type (W) and fully silenced (S) plants was digested with McrBC at the indicated times and the *TDET1* transgene subsequently amplified by PCR. A methylated control DNA is also shown. The DNA used for methylation analysis was derived from plants from T4 generation, and results obtained were identical to those found in the T3 generation (data not shown).

3.4 Discussion

Although DET1 has been studied in both *Arabidopsis* and tomato (Mustilli et al., 1999; Pepper et al., 1994), its function is only partially understood (Benvenuto et al., 2002; Schroeder et al., 2002). In an attempt to obtain new insights into the role of this protein in a developmental context in tomato, several transgenic tomato plants have been generated containing a range of *TDET1* constructs under the control of three different promoters. When phenotypes were visible they were invariably characteristic of exaggerated light sensitivity. Molecular examination of these plants indicated in all cases that the phenotypes were a result of suppression of endogenous *TDET1* expression rather than the overexpression of wild-type or dominant negative versions of *TDET1*. Subsequent analyses showed that these phenotypes were caused by PTGS of *TDET1*.

Reiteration of light hyper-responsive *high pigment* phenotypes by suppression of *TDET1* expression is consistent with the hypothesis that *hp2*-mutant phenotypes are caused by reductions rather than alterations in TDET1 activity (Mustilli et al., 1999). This was further confirmed by complementation of the *hp2* mutant with a wild-type *TDET1* gene (Figure 3.4b).

The importance of DET1 function at all stages of development was evident from the severity of phenotypes generated by suppression of its expression (Figure 3.1). On the contrary, plants overexpressing high levels of the transgene had wild-type phenotypes in all cases studied. These findings indicate that plants are very sensitive to reductions in DET1 levels but not to increased levels, perhaps because DET1 levels are already saturated. Similarly, in *Arabidopsis* overexpression of *DET1* did not appear to confer any altered phenotypes (Schroeder et al., 2002).

Whereas *DET1* silencing was often lethal when it occurred at a relatively early stage during development (Figure 3.1g), this was never the case in fruits. Even in cases of extreme silencing in fruits, the fruits remained healthy in spite of their extreme pigmentation. This finding could indicate that DET1 activity regulates essential processes in the vegetative parts of the plant but not in the fruits. In tomato fruits it is known that phytochromes can regulate pigment accumulation (Alba et al., 2000), although how it does so is unclear. They may simply regulate the expression of genes encoding pigment biosynthetic enzymes (Giuliano et

al., 1993) or they may regulate plastid compartment size within the cells of the fruit (Cookson et al., 2003). The transgenic plants generated in this study could be useful for examining these possibilities. Overexpression of either mutant (*hp2* and *hp2^j*) or truncated versions of the *TDET1* gene generated the strongest silencing of *TDET1*, and caused the most severe developmental defects. Hence, it appears that expression of aberrant transgenes was much more efficient at inducing PTGS than were wild-type sequences. The fact that a sequence with a single point mutation (*hp2^j*) generated such severe phenotypes may be an indication of the impressive capacity of the plant cell's surveillance system to detect abnormal RNAs (Vaucheret and Fagard, 2001). Alternatively, the weaker phenotypes generated in *e35S::TDET1* transgenic plants may be a result of the production of some viable DET1 protein.

The severity of phenotypes in plants containing the 5'-terminal and 3'-terminal constructs was also different, in that the 3'-terminal constructs were able to generate much stronger phenotypes than the 5'-terminal constructs. Furthermore, severity of phenotype was clearly correlated with the amounts of 25 nt siRNAs, indicating that production of dsRNAs was more efficient from 3' sequences of the *TDET1* gene. Similar results were reported by Han and Grierson (2002), who found higher amounts of siRNAs derived from the 3'-terminus of a silenced *polygalacturonase* gene. These authors concluded that siRNAs are synthesized preferentially from the 3-region of a transgene.

Because loss of DET1 activity results in dark green phenotypes, these plants are useful to follow the systemic spread of silencing within a plant. This was most clear in the fruits, and one such example is shown in Figure 3.4a. When silencing was triggered within the mature fruits of one truss, a silencing signal appeared to travel to other fruits of the same truss, particularly the younger ones, and a clear gradient of phenotypes was observed, which was also confirmed by RT-PCR (data not shown). In many cases the fruits were not completely dark green, but appeared blotchy and remained so for several weeks and often up to complete maturity. On the contrary, in the vegetative parts of the plant, silencing appeared to spread to neighbouring parts much more quickly, generally towards the younger upper leaves. This may be because the systemic silencing signal travelled faster through phloem than from one cell to another. An alternative explanation could be that because tomato fruits are sink organs, signals tend not to be released efficiently from them. It has been proposed that phloem-

mediated transmission of PTGS may be dependent upon the generation of 25 nt siRNAs, whereas long-range cell-to-cell transmission may require only 21 nt siRNAs (Hamilton et al., 2002; Himber et al., 2003). Assuming that long-range cell-to-cell transmission is responsible for our blotchy fruit phenotypes, the fact that only siRNAs of the 25 nt size class were observed does not support this hypothesis.

The silencing phenotype was not inherited from generation to generation, but had to be acquired in each successive generation (Figure 3.5). The wild-type phenotypes of progeny seedlings derived from silenced plants suggest that silencing was relieved during sexual reproduction and that siRNAs did not enter the seeds. A previous study of PTGS in tobacco reported similar observations (Mitsuhara et al., 2002).

Interestingly, the results presented in this Chapter indicate that PTGS of *TDET1* acts only at the level of RNA degradation and not at the genomic level, because no evidence for hypermethylation of either the transgene or the endogene was found (Figure 3.7). This indicates that PTGS does not require DNA methylation per se, which had been previously inferred from other studies (Wang and Waterhouse, 2000). The lack of any evidence for DNA hypermethylation is nonetheless curious considering the increased penetrance of the silencing phenotype in successive generations (Figure 3.5), and demonstrates a lack of correlation between 25 nt siRNA production and DNA hypermethylation (Himber et al., 2003).

Finally, these results may have implications for the genetic improvement of plant nutritional quality. Tomatoes are known to be a rich source of health-promoting carotenoids and vitamins and are a multi-billion dollar global industry. In *TDET1*-silenced fruits, lycopene and β-carotene levels were increased dramatically. These results indicate that manipulation of *TDET1* levels has promising biotechnological potential. However, the reduced yields and collateral negative effects on the vegetative parts of the plant must first be eliminated. Use of a fruit-specific promoter in combination with a *TDET1* silencing construct may provide an ideal strategy, although the fruit-specific E8 promoter used in this study (Deikman et al., 1992) was clearly ineffective (Table 3.1). Alternative promoters or a more efficient silencing construct (e.g., containing inverted repeats; Wesley et al., 2001) may therefore be necessary.

In conclusion, the results presented here clearly support the idea that DET1 is an important negative regulator of light signalling that is required throughout the plant life cycle. These results also indicate that DET1 may function differently during plant and fruit development, being essential in the former but not in the latter. Finally, it appears that the role of DET1 may be to ensure quantitatively appropriate responses to incoming light signals and that DET1 levels in wild-type cells are already sufficient for this, although *DET1* is expressed at extremely low levels. The mechanistic significance of these findings in the context of the proposed role of DET1 in chromatin remodelling (Benvenuto et al., 2002) will be a major challenge to address in future research.

3.5 Materials and Methods

3.5.1 Plasmid Construction

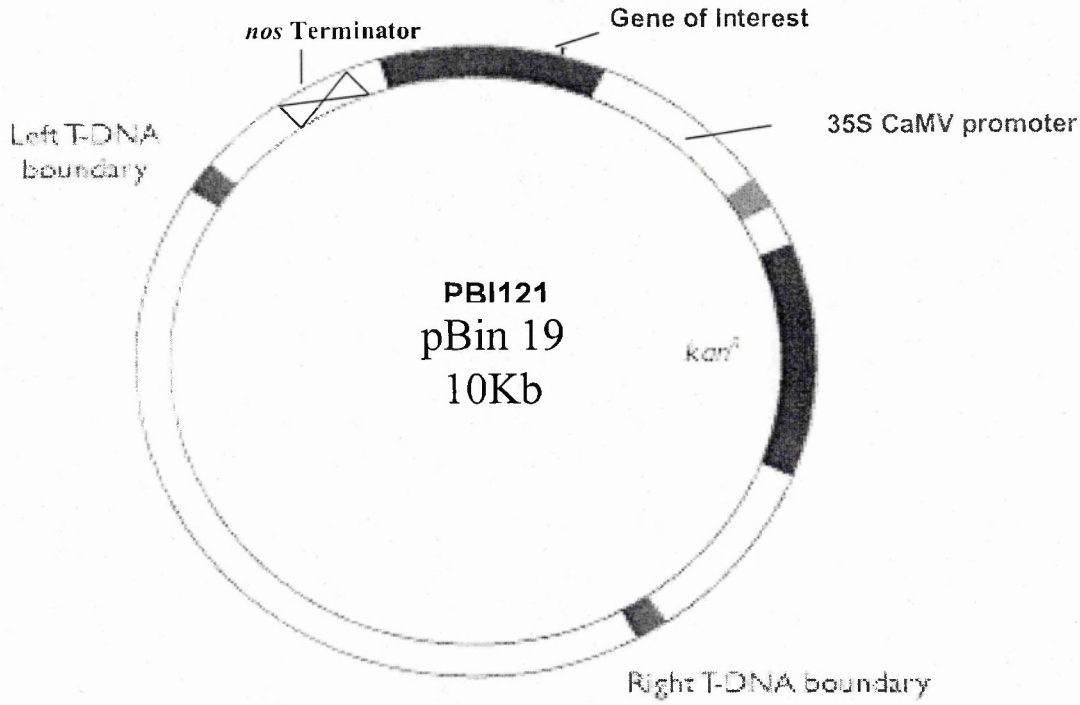


Figure 3.8. Schematic representation of the plasmid pBI121 used to generate all the expression cassettes.

All expression cassettes were made in plasmid pBI121 (Figure 3.8) (Bevan, 1984), which is a binary vector commonly used for *Agrobacterium tumefaciens*-mediated transformation of plant tissues (McCormick, 1991;Yoder et al., 1988). This vector contains *NPT II* as a marker gene encoding the *neomycin phosphotransferase* II gene for kanamycin resistance driven by the CaMV 35S promoter (Benfey and Chua, 1990). An additional 35S promoter is located upstream of a cloning site for the insertion of genes of interest. The full-length tomato *DET1* gene (*TDET1*) (Mustilli et al., 1999) was first cloned in pBS-KS to generate suitable restriction sites and was subsequently cloned in pBI121 as a transcriptional fusion between the 35S promoter and the nos terminator as a *BamH1-Sac1* fragment. This construct was denoted *35S::TDET1*. Similarly, the *hp2* and *hp2^j* mutant versions of *TDET1* (Mustilli et al., 1999) were cloned first in pBS-KS and then in pBI121. These constructs were denoted *35S::hp2* and *35S::hp2^j*. Two other constructs were designed to express 5'-terminal and 3'-terminal truncated versions of *TDET1*. The 5'-portion of the *TDET1* gene up to nucleotide 1254 was cloned in pBI121 after first adding a stop codon by PCR at the end of the sequence. This construct was denoted *35S::TDET1-5'*. A 3'-portion of *TDET1* was cloned, starting from nucleotide 750, after adding a start codon at the 5'-end. This construct was denoted *35S::TDET1-3'*. The primers used for generating the 5'-terminal construct were 5'-GCG GCGAGCTCTTAAAATGGTCGCTGAACAG-3' and 5'-TTAAAAATGGTCGTCGAACA G-3' (to add the stop codon for *35S::TDET1-5'*). The primers used for the 3-terminal construct were: 5'-ATG TTCCACCTTTTGAGGTTGGTG-3' (to add the start codon for *35S::TDET1-3'*) and 5'-GCGGCGAATCCATGAGGTTGGTG-3'. The *TDET1* gene was also inserted in the antisense orientation to generate *35S::TDET1-AS* construct. In addition to the 35S-based antisense construct, we also generated a construct with the fruit-specific E8 promoter (Deikman et al., 1992). A 1.2 kb E8 promoter fragment was cloned in pBI121 between HindIII and BamH1 sites using 5'-GGGGAAGCTTTTTCACGAAATCGGCCCT TA-3' and 5'-CCCGGATCCTTCTTTTGCACTGTGAATGATTAG-3', and the full length *TDET1* coding sequence was fused in either sense or antisense orientations downstream of the promoter as *BamH1-Sac1* fragments. These constructs were denoted *pE8::TDET1* and *pE8:: TDET1-AS*, respectively. For stronger expression, the e35S promoter, which contains a duplicated 35S promoter sequence without the leader sequence was used. These constructs were made in an analogous fashion. All the constructs were introduced into *A. tumefaciens* strains LBA 4404 or ABI for tomato plant transformation. A summary of constructs and results obtained is shown in Table 3.1.

3.5.2 Plant Transformation

To generate transgenic tomato plants, cotyledons from 1 to 2-week-old seedlings were used for protocols as described by McCormick (1991) and Yoder et al. (1988). As wild-type backgrounds Money Maker and T56 genotypes were used. T56 is a commercial processing tomato variety. The Money Maker genotype was transformed as described by McCormick (1991) whereas T56 was transformed essentially according to Yoder et al. (1988). For T56, tomato seeds were sterilized using 2% commercial bleach and germinated on MSSV medium (Fillatti et al., 1987). Four to 7-day-old cotyledons were excised and placed onto freshly prepared tobacco feeder plates, prepared by decanting 1–2 mL of tobacco cells in suspension culture onto 2Z medium (Thomas and Pratt, 1981). After 48 h the cotyledons were immersed for 5 min in an overnight culture of *A. tumefaciens* strain ABI diluted to an OD600 of 0.1. After 24 h the explants were plated onto 2Z medium containing 350mg/mL carbenicillin and 100mg/mL kanamycin sulphate. Excised shoots were placed onto rooting medium containing 50mg/mL kanamycin. Primary transformants were screened for the presence of the transgene by PCR and were transferred to soil. Adult plants were grown under shaded greenhouse conditions (25 ^{0}C day/17 ^{0}C night). It was observed that regenerated callus from transformation with some of the constructs was dark green, and that some of the regenerated plants were darker green and produced dark green immature fruit phenotypes (see text for details).

3.5.3 Molecular Characterization of Plants

DNA was isolated from young leaves using the CTAB method (Dellaporta et al., 1983). For methylation analysis, 10 μg of DNA was digested overnight with *AluI*, *BglII+EcoRI*, *DdeI*, and *Sau3AI* restriction enzymes. The digested DNA was run on 1.0% agarose gels and blotted to Hybond N membranes (Amersham Pharmacia Biotech, UK) overnight using 20X SSC. Filters were hybridized with random-primed full-length *TDET1* probes labelled with 32^{α}-P-CTP. Hybridizations were carried out overnight at 60 ^{0}C in Church and Gilbert buffer (7% SDS, 0.5 M NaPO4 pH 7.2 and 1 mM EDTA) (Church and Gilbert, 1984). The washes were carried out using 0.1% SDS and 1X SSC at the same temperature twice for 10 min each.

For isolation of RNA, tomato leaves or pericorp of immature fruits (0.2g) were ground to a fine powder in liquid nitrogen and subsequently extracted using the hot phenol method (DellaPenna *et al.*, 1986). To the grounded powder 5mL of extraction buffer (100mM tris pH8.0, 10mM EDTA and 1%SDS) and 5mL of Phenol mix, which was preheated to 80 0C, was added and vortexed vigorously. The samples were spun at 5000 rpm for 10min and the aquous phase was carefully removed into a new folcon tube. The samples were extracted once with Chloroform isoamyl alcohol (24:1), added eauqal volume of 4M Lithium chloride and kept at -80 ^{0}C for 2 hours. The RNA was washed with 70% EtOH and suspended in 200-400 µl of DEPC water RNA (10 µg) was loaded onto formaldehyde gels and blotted to Hybond N membranes using 10X SSC overnight. Hybridization was carried out overnight using an 32$^\alpha$-P labelled *TDET1* probe with Church and Gilbert buffer. The washes were performed in 0.1% SDS and 1X SSC twice for 5 min each. Blots were stripped using hot SDS (1%) and re-probed with an 32$^\alpha$-P-labelled *CAB6* probe (Piechulla et al., 1991). For isolating low molecular weight RNA, the protocols described by Hamilton and Baulcombe (1999) with small modifications was used. Low molecular weight RNA was separated from high molecular weight RNA using 10% PEG8000 and 0.5 M NaCl; 50 µg of low molecular weight RNA was run on 15% polyacrylamide gels containing 7 M urea and electoblotted onto Hybond NX membranes. In vitro-transcribed 32$^\alpha$-P-labelled full-length *TDET1* antisense RNA was used as a probe and hybridizations were carried out at 60 ^{0}C in Church and Gilbert buffer for 24 h. The blots were washed with 0.1% SDS and 20 mM NaPO4 twice for 10 min each. For RT-PCR analysis, poly A mRNA was isolated using Dynal mRNA extraction kits and protocols recommended by the manufacturers (Dynal Inc., Norway). poly A RNA (100 ng) was used to synthesize first-strand cDNA using the Invitrogen Superscript First Strand Synthesis kit (Invitrogen Corporation, Carlsbad, USA). From this 2 µL of first strand cDNA was used for *TDET1* amplification using specific primers 5'-GTATGATTCACTAGTTTA-ATGCTGCTGAAAG-3' and 5'-CCCATACTAGTCGTCTTGGCACTCTATCAAG-3'. As a control for normalization, the HY5 gene (Oyama et al., 1997) was amplified using specific primers 5'-ATGCAAGAGCAAGCGACGGTTCTAT-3' and 5'-GTCCACGTGTCCTTCC-CTCCTTCA-3'. For McrPCR, 2 µg of genomic DNA extracted from leaves of transgenic plants was digested with 30 units of McrBC enzyme as suggested by the supplier (New England Biolabs). DNA was incubated at 37 ^{0}C for 25 min and 8 h. As a control, pre-methylated DNA from the supplier of the enzyme was used. After digestion the enzyme was heat inactivated by incubating at 65 ^{0}C for 20 min. DNA (50 ng) was then used to amplify the

TDET1 transgene using primers 5'-GTATGATTCACTAGTTTAATGCTGCTGAAAG-3' and 5'-CCCATACTAGTCGTCTTGGCACTCTATCAAG-3' for the full length transgene and 5'-GAAAGCAGCCGTTGCT-3' and 5'-CCCATACTAGTCGTCTTGGCACTCTATC-3' for the *TDET1-3'* construct. After 25 PCR cycles the reaction was analysed on 1% agarose gels.

3.5.4 Biochemical Analysis of Fruits

For chlorophyll extraction, approximately 0.05 g of immature fruit peel was weighed and put in 5 ml DMSO. The tubes were incubated at 65 ^{0}C for 48 h in the dark. Chlorophyll content was determined by measuring absorbance at 649 and 665 nm and concentrations were calculated using the equations for ethanol published by Lichtenthaler and Wellburn (1983). The analysis was repeated thrice, in each case with five fruits from each phenotype.

Fully red ripe greenhouse fruits were harvested between the 15 and 20 day post-breaker stage for biochemical analysis. Carotenoids were extracted from tomato puree using 15 mL of acetone/methanol (2:1) and 4 ml hexane. Following addition of approximately 21 mL cold saline water, samples were shaken vigorously and then centrifuged into separate phases. An aliquot of the hexane phase was taken and analyzed on an HP 1050 system configured with a diode array detector. The carotenoids were separated on a Whatman Partisil 5 ODS-3 column using a solvent mix of 81.7% acetonitrile, 9.6% methanol, 5.4% isopropyl alcohol and 3.3%MQ water. Lycopene was detected at 504 nm and β-carotene at 450 nm. The Brix (Soluble Solids) value was measured on a Bellingham & Stanley RFM-91 refractometer using filtered tomato puree. Sugars were extracted from tomato puree using ethanol (final concentration 80%). Following centrifugation, an aliquot of the extract was removed and analysed on an HP 1050 HPLC system configured with a refractometer. The sugars were separated on a Whatman Partisil PAC column using a solvent mix of 87% acetonitrile and 13% water. This biochemical analysis was done in collaboration with The Seminis Vegetable Seeds Inc (Woodland, CA-USA).

CHAPTER 4

Fruit-Specific Suppression of *DET1* Expression Results in Tomatoes with Enhanced Nutritional Value

4.1 Summary

Tomatoes are a principal dietary source of carotenoids such as lycopene and β-carotene, both of which have been shown to be highly beneficial for human health. The overexpression of genes encoding carotenoid biosynthetic enzymes have allowed the generation of tomatoes with improved carotenoid content. However, because carotenoid biosynthesis involves the products of several genes, overexpression of just one may result in only minimal increases in nutritional value. Furthermore, use of genes from other organisms is a major public concern for the commercialization of transgenic plants. Here one attempt is described to increase fruit carotenoid content by suppression of an endogenous regulatory gene, *TDET1*, in a fruit-specific manner using fruit-expressed promoters combined with post transcriptional gene silencing (PTGS) technology. DET1 is a negative regulator of light signal transduction involved in the regulation of a range of light-dependent genes, including those for carotenoid biosynthesis. Several independent transgenic lines with *TDET1* constructs designed to induce PTGS under the control of three different fruit-expressed promoters have been generated. Molecular analysis indicated that *TDET1* transcripts were indeed specifically degraded in fruits. Furthermore, more than 50 % of the transgenic lines showed dark green immature fruit phenotypes while the plants remained healthy. In mature fruits, lycopene showed a 7-fold range of increase and a 3-fold average increase among lines. Significant increases were also observed for β-carotene, and levels were increased on average by 7-fold in transgenic lines. Transgenic plants showed very little yield losses and tomato juice brix values were similar to values of wild-type fruits. These results therefore suggest that manipulation of a light regulatory gene such as *DET1* can influence the production of several phytonutrients, and provide a novel example for the use of tissue-specific gene silencing to improve the nutritional value of plant-derived products.

4.2 Introduction

Plant-based food offers a diverse mixture of nutrients that are essential for human nutrition and which can contribute to the promotion of good health. It has been suggested from epidemiological studies that increased consumption of fruits and vegetables is correlated with a reduced risk of several diseases, including cancer and cardiovascular disease (Department of Health: *Report on Health and Social Subjects* 48, HMSO, London 1998). There is considerable interest in the development of food products rich in vitamins and carotenoids

because it is generally thought that they will be more beneficial to human health than dietary supplements (Cooper et al., 2004). Although conventional breeding is one means of achieving this goal, the genetic diversity available within existing germplasm for any given crop will limit the extent of improvement. Transgenic approaches offer a powerful alternative for improving nutritional value in plants although there is currently a great deal of public concern about their use in contemporary agriculture, in particular those containing genes derived from organisms other than plants.

Tomato cultivation and processing are multi-billion dollar industries worldwide (US Department of Agriculture. *Agricultural Statistics*, 141-175, 1991). Tomato fruit and derived products are the principal dietary sources of lycopene and also contain large amounts of β-carotene. Increased lycopene gives the fruit a more appealing colour, improves the quality of paste, and has proven nutritional value as an antioxidant (Miller et al., 2002). Increased lycopene in the diet is associated with reduced heart attack rates and is also a promising cancer chemopreventative, particularly for prostate cancer (Miller et al., 2002; Kucuk et al., 2002; Heber and Lu, 2002). Beta-carotene is the most potent precursor of vitamin A, deficiency in which can cause xerophthalmia, blindness, and premature death. Vitamin A deficiency is the most common dietary problem affecting children worldwide, and UNICEF has estimated that improved vitamin A nutrition could prevent up to 2 million deaths annually among children aged between one and four years (Humphrey et al., 1992).

Several attempts have been made to improve the carotenoid content of tomato products. For example, overexpression of a bacterial gene encoding phytoene desaturase was found to increase β-carotene levels but not lycopene (Romer et al., 2000), whereas overexpression of a bacterial phytoene synthase resulted in moderate increases in both β-carotene and lycopene (Fraser et al., 2002). Approaches such as these based on the use of genes encoding biosynthetic enzymes tend therefore to increase only one or a few metabolites. Furthermore, the fact that bacterial genes have been used may prove to be an insurmountable obstacle for the commercialization of such transgenic plants.

One strategy to obtain more general increases in several metabolites could be to modulate regulatory genes whose products control flux through entire biosynthetic pathways (DellaPenna, 2001). These genes would be of plant origin and so such strategies may also be

more acceptable to the general public. In addition to the well-documented role of ethylene in fruit ripening (Giovannoni, 2001), it has recently emerged that genes encoding components of the light signal transduction machinery can influence tomato fruit quality (Alba et al., 2000) and so may represent promising genetic tools to improve nutritional value. *DE-ETIOLATED1* (*DET1*) is one such regulatory gene, which represses several signalling pathways regulated by light (Schäfer and Bowler 2002). Mutations in this gene are responsible for *high pigment 2* (*hp2*) phenotypes in tomato, characterized by exaggerated photoresponsiveness (Mustilli et al., 1999). Light-grown *hp2* mutants display high levels of anthocyanins, are shorter and darker than wild-type plants and have more deeply pigmented fruits. The higher pigmentation of mature fruits from these mutants is due to elevated levels of flavonoids and carotenoids (Mustilli et al., 1999; Yen et al., 1997).

Constitutive silencing of the tomato *DET1 (TDET1)* gene in tomato was shown in Chapter 3 to cause elevations in β-carotene and lycopene in mature fruits, although severe developmental defects such as reduced stature, bushiness, and dwarfing were also observed. Such phenotypes are also observed in *hp2* mutants, which is why the mutation has not been successfully exploited by breeders. In order to harness the positive effects of *TDET1* gene suppression in fruits without the collateral negative effects on plant growth, its expression should be modulated only within the fruits. Therefore fruit-specific *TDET1* silencing constructs were generated, and this Chapter shows that suppression of *TDET1* expression specifically in the fruits indeed enhances their carotenoid content, with only minimal effects on plant growth and fruit yield.

4.3 Results

4.3.1 Constructs Used for Generation of Transgenic Plants.

In order to suppress *TDET1* gene expression specifically in fruits three different fruit specific promoters were used, denoted P119, 2A11 and TFM7. It was hypothesized that early-stage expression during fruit development could be necessary because the late-expressing E8 promoter (Deikman et al., 1998) was found in Chapter 3 to be ineffective and because the *hp2* mutation has strong effects in immature fruits (Mustilli et al., 1999). All three promoters are known to be expressed early during fruit development (Pear et al., 1989; Santino et al., 1997).

Promoter P119 is expressed from the earliest stages in green fruit through to red ripe stages, with increased levels during ripening (Dunsmuir et al., 1997). Promoter 2A11 is expressed at high levels in ripening fruits, but also transiently in 2-3 cm-sized green fruits (Santino et al., 1997). The TFM7 promoter is expressed mainly during immature green fruit development and becomes inactive as green fruits reach full size (Santino et al., 1997).

Inverted-repeats *TDET1* constructs were generated under the control of the three fruit-specific promoters (Figure. 4.1) because it has been previously shown that the utilization of such repeats can greatly enhance gene silencing in both plants and animals (Smith et al., 2000; Wesley et al., 2001). The constructs were introduced into tomato plants using *Agrobacterium tumefaciens*-mediated transformation (Yoder et al., 1998). Several independent lines were generated, screened by PCR, and grown to maturity under greenhouse conditions. Subsequently progeny were grown from self-fertilized seeds and analyzed by PCR of genomic DNA for the presence of the transgenes. Generation of constructs and transgenic plants were carried out by Seminis Vegetable Seeds Inc (Woodland, CA-USA).

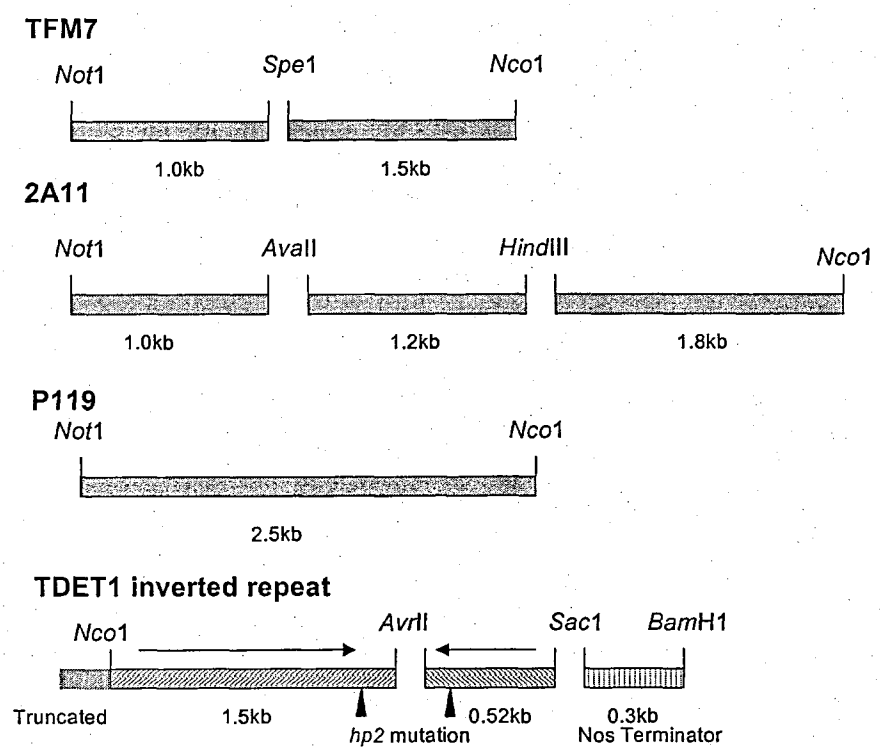


Figure 4.1. Schematic representation of DNA fragments used to make chimeric constructs with fruit-specific promoters and *TDET1* (*hp2* mutant version) inverted-repeats. For more details see Materials and Methods.

4.3.2 Visual Phenotypes of Transgenic Plants.

The vast majority of transgenic plants appeared normal and healthy. Interestingly, however, many of them developed dark green immature fruits that subsequently became deep red at the mature stage (Figure 4.2), reminiscent of *hp2* mutants (Mustilli et al., 1999). With the P119 promoter construct the dark green fruits were mainly observed on the lower trusses and fruits had a grainy appearance (Figure 4.2b). With the 2A11 promoter the dark green fruits uniformly developed on all trusses all the way up the plant. Furthermore, phenotypes were not grainy but more uniformly dark green (Figure 4.2c). With the TFM7 promoter, dark green fruits were also observed on trusses of all ages and were again not grainy (Figure 4.2d). Immature fruits of plants containing the 2A11 construct were generally even darker than the fruits of transgenic plants containing the other two promoter constructs. In spite of these minor differences, the red-ripe fruits from plants containing all three promoter constructs appeared dark red compared to wild-type fruits, indicative of higher lycopene levels (Figures 4.2e, f).

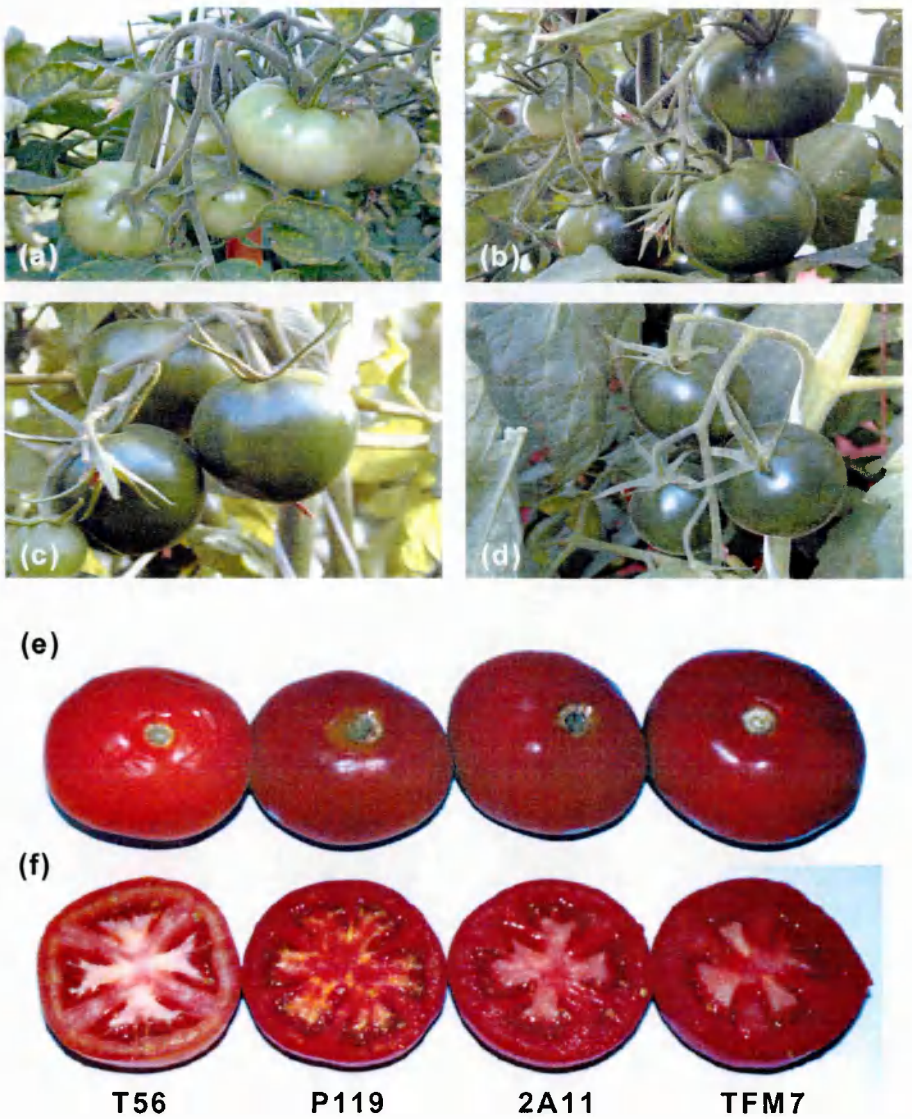


Figure 4.2. Fruit-specific phenotypes of T_2 generation transgenic plants containing different promoter constructs. (a) Immature fruits from T56 (wild type) plants, (b) Immature fruits from plant containing *P119::hp2-IR::nos*, (c) Immature fruits from plant containing *2A11::hp2-IR::nos*, (d) Immature fruits from plant containing *TFM7::hp2-IR::nos*, (e) Fully red-ripe fruits from wild-type plants and transgenic plants containing the three promoter constructs, (f) Cross-sections of fruits shown in (e).

4.3.3 Molecular Analysis of Transgenic Plants.

The fruit-specific phenotypes of transgenic plants containing all three promoter constructs suggested strongly that *TDET1* gene expression had been suppressed specifically in the fruits. Due to the inverted-repeat design of the constructs, this suppression had most likely been achieved by post-transcriptional gene silencing (PTGS) (Fagard and Vaucheret, 2000). A diagnostic feature of PTGS is the degradation of target mRNAs into low molecular weight products (Hamilton and Baulcombe, 1999). Therefore endogenous *TDET1* mRNA abundance in the transgenic plants was examined using a range of techniques. Real-time quantitative RT-PCR analysis of RNA extracted from fruits and leaves of all plants examined indicated that *TDET1* mRNA in the fruits was much less abundant than in the leaves (Figure 4.3a). In RNA extracted from leaves, the relative amounts of *TDET1* mRNA levels were however much the same as in wild-type plants. To further analyze *TDET1* mRNA levels, semi-quantitative RT-PCR was performed with RNA isolated from both fruits and leaves of the same plant. Full length *TDET1* mRNA was amplified using primers which cover the complete coding sequence of the gene and the *HY5* gene was amplified from the same cDNA samples as a control to standardize cDNA synthesis. These analyses revealed that *TDET1* mRNA levels in the fruits were drastically reduced compared to leaves of the same plants and demonstrated that this was the case for all three of the fruit-specific promoter constructs (Figure 4.3b). This was not observed in non-transformed plants, where *TDET1* mRNA levels in different parts of the plant were similar, and were present at low levels at all stages of plant development (Figure. 4.3c). These results contrast with those obtained previously from plants containing constitutive *35S::TDET1* PTGS constructs, where *TDET1* mRNA degradation was observed in all tissues examined (Chapter 3).

To better estimate the extent of *TDET1* gene silencing in fruits northern blots of low molecular weight RNA was done in order to detect siRNAs, which are a hallmark of PTGS (Hamilton and Baulcombe, 1999). *TDET1*-derived siRNAs of 25 nucleotides in size were indeed detected in fruits but not in the leaves (Figure 4.3d). This was the case in all lines expressing the three different fruit-specific promoters. This result conclusively demonstrated the successful silencing of the *TDET1* gene in the fruits and revealed that gene silencing did not occur or did not spread to other parts of the plant. Because loss of TDET1 function causes darker green foliage and bushy phenotypes (Chapter 3), it was easy to assess whether its

expression had been modulated also in the vegetative parts of the plant. This was not observed in any of the transgenic plants, neither in the primary transformants nor in subsequent generations (data not shown).

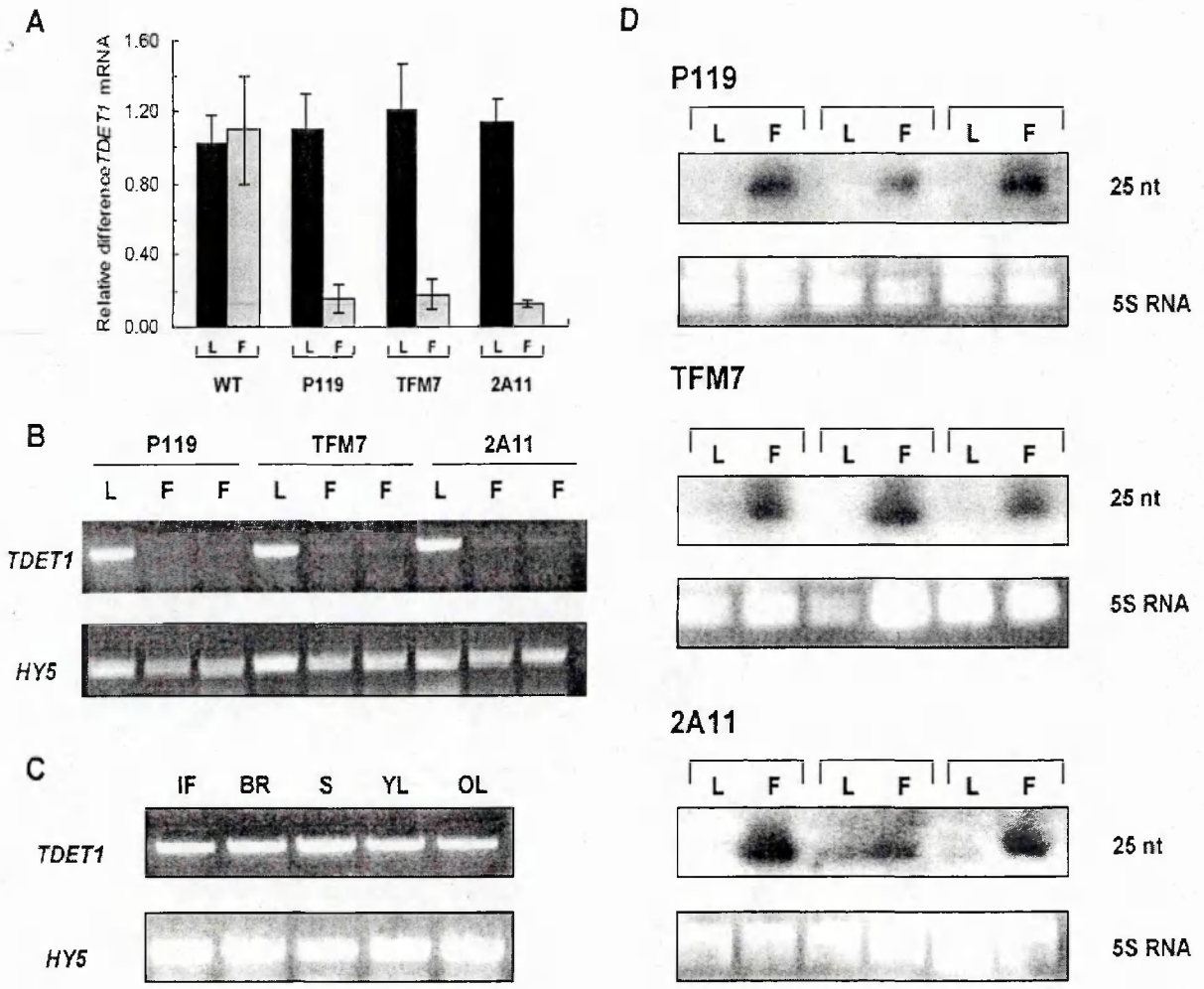


Figure 4.3. Analysis of *TDET1* mRNA in transgenic plants containing fruit-specific promoter constructs. A: Real time RT-PCR analysis of leaves (L) and mature green fruits (F) from T56 (wild type) and T3 generation transgenic plants P119, TFM7 and 2A11. *TDET1* mRNA abundances are shown relative to mRNA levels in leaves of wild-type plants. Each bar represents 3 repetitions from each RNA sample (derived from pools of 3 fruits or leaves per plant, and from 2 individual plants for each genotype). Error bars representing standard deviations are shown in each case. B: Semi-quantitative RT-PCR analysis of *TDET1* expression in leaves (L) and mature green fruits (F) from T3 generation plants transformed with fruitspecific promoter constructs. Full length *TDET1* was synthesized from RNA extracted from leaves and fruits from the same plants. The *HY5* gene was used as control for cDNA synthesis. C: Semi-quantitative RT-PCR analysis of *TDET1* mRNA levels in different tissues from wild-type (T56) plants. IF: immature green fruit, BR: breaker stage fruit, S: stem, YL: young leaf, OL: old leaf. The *HY5* gene was used as control for cDNA synthesis. D: siRNA analysis of mature green fruits (F) and leaves (L) from transgenic plants containing fruit-specific promoter constructs. *TDET1*-derived siRNAs were 25 nt in length. 5S rRNA is shown as loading control. In each panel, the results from 3 independent T3 plants are shown.

4.3.4 Organoleptic Analysis of Red-Ripe Fruits.

Because TDET1 is an important regulator of light signaling and acts as a repressor, many genes are up-regulated upon loss of TDET1 activity. For example *CHS* expression is higher in leaves and *CAB* expression levels are greatly increased in immature fruits of *hp2* mutants (Mustilli et al., 1999). Furthermore, mature fruits from *hp2* mutants contain increased levels of carotenoids (Mustilli et al., 1999). Chemical analysis performed by Seminis Vegetable Seeds Inc (Woodland, CA-USA) of fruit samples selected from transgenic plants containing all three promoter constructs revealed that both lycopene and β-carotene were present at much higher levels than in wild-type fruits (Figure 4.4a, b).

All three promoter constructs generated much higher levels of lycopene than in wild-type fruits. With the P119 promoter, average lycopene levels were increased 1.7-fold (94.4 ppm) compared to wild-type fruits (55.9 ppm), but in some lines lycopene levels were more than 3-fold higher (175.5 ppm). The 2A11 promoter construct increased lycopene levels to a lesser extent (87.5 ppm), although in some lines values were increased by almost 3-fold (157.0 ppm). In mature fruits from plants containing the TFM7 promoter construct, average levels were increased by almost 2-fold (106.5 ppm). In fruits from some lines the levels were 3-fold higher (171.3 ppm).

β-carotene levels in fruits from transgenic lines showed even greater increases (Figure 4.4b). The highest levels were achieved with the P119 promoter construct, and levels were increased almost 6-fold (13.4 ppm) compared to wild-type fruits (2.4 ppm). In some lines the amount of β-carotene was increased by almost 10-fold (23.4 ppm). With promoter 2A11, β-carotene levels averaged 2.5-fold higher levels (6.0 ppm), although in some lines maximum levels were almost 5-fold higher (11.4 ppm) than in wild-type fruits. With the TFM7 construct, β-carotene levels averaged a three-fold increase (7.2 ppm), but some lines had more than a 7-fold increase (17.4 ppm). In summary, the P119 promoter construct provided the most dramatic increases in β-carotene, which was also evident from visual inspection of fruits from these plants, which were more evidently orange (Figure 4.2f).

Vitamin A units were similarly increased in fruits from transgenic lines. The maximum levels were obtained with the P119 promoter, with as much as a 10-fold increase (39.0 IU/g)

compared to average values in fruits from wild-type plants (4.0 IU/g). The average between lines was a little less than 6-fold (22.3 IU/g). With the 2A11 promoter construct the average values were 2.5-fold increased (10.0 IU/g) and in some lines the maximum levels were close to 5-fold higher (19.1 IU/g). With the TFM7 promoter construct an average increase of 3-fold (12.1 IU/g) was observed, but in some lines levels were 7-fold higher (29.0 IU/g).

Fruit weight averaged 114 grams. Fruit weights from wild-type plants (average, 126 g/3 fruits) and transgenic lines containing the P119 promoter construct (average, 129 g/3 fruits) were very similar, whereas yields from lines containing the 2A11 and TFM7 constructs were slightly reduced (average values of 106 g and 98 g/3 fruits, respectively), even though the distributions of fruit weight from each population varied between 100 and 150 grams per 3 fruits, which was similar to that found from wild-type plants (Figure 4.4c). Tomato juice brix (average 4.6 g/100g) and total sugar content (average 2.2 g/100g fresh weight) were not significantly different between wild-type and transgenic lines (data not shown).

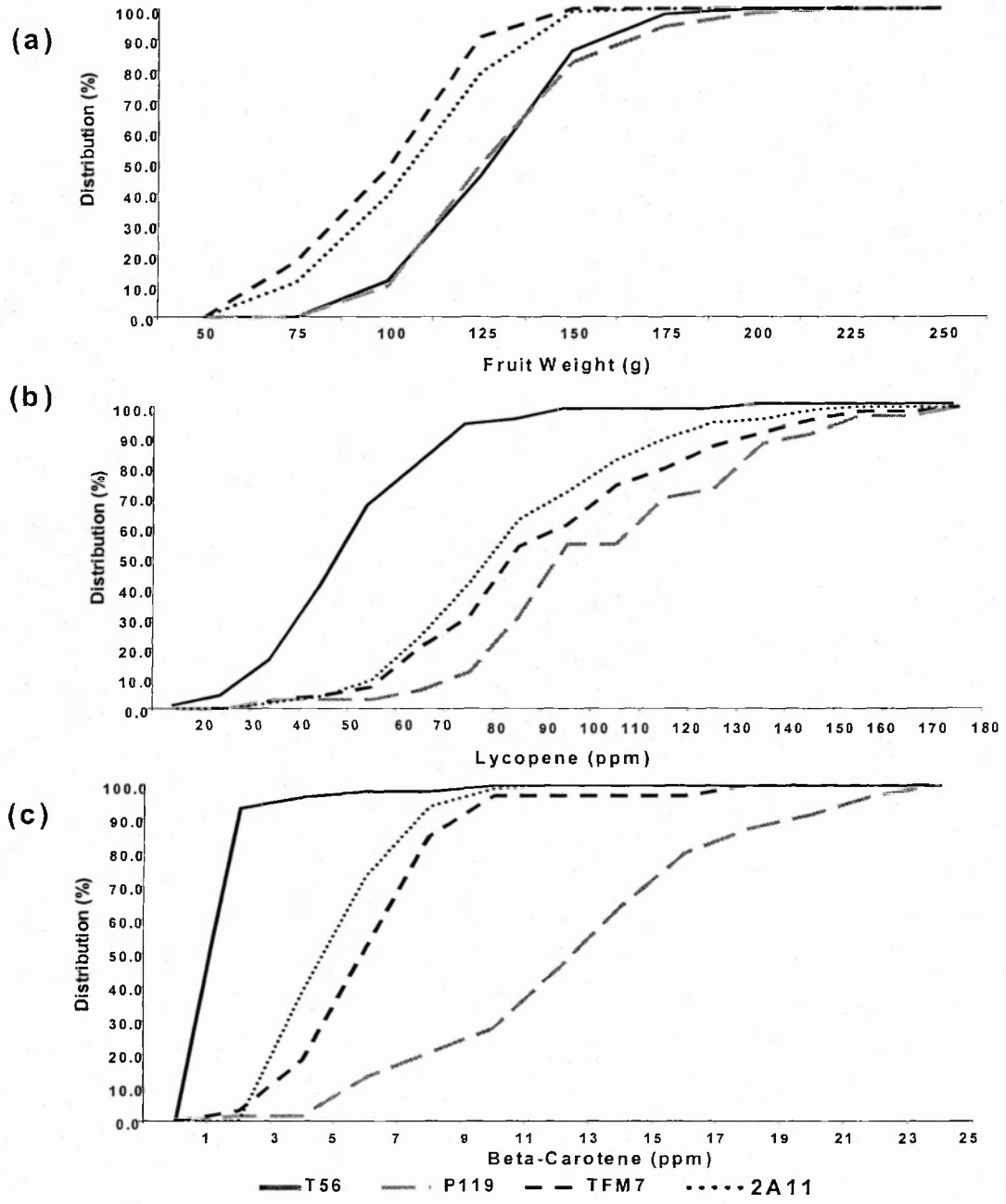


Figure 4.4. Quantification of fruit weight and carotenoid content in red-ripe fruits from T56 (wild type) and T_3 generation plants containing fruit-specific promoter constructs. Each plot shows cumulative distributions (in percent) as an indication of the phenotypes conferred by each of the transgenes in all of the plants examined. (a) Fruit weight, (b) Lycopene distributions, (c) Beta-carotene distributions (from samples of 3 fruits per plant). Each analysis is derived from results obtained from 59 wild-type plants, 69 P119 construct plants, 96 2A11 construct plants, and 33 TFM7 construct plants. Lycopene values are generally low because plants were grown in the winter-spring season. A similar trial performed in the summer season with T_2 generation plants revealed very similar differences between constructs although lycopene levels were generally higher in fruits from all plants (data not shown).

4.3.5 Stability of Phenotypes.

Visual phenotypes were observed to be highly reproducible in different sister lines (data not shown). All lines showing fruit phenotypes in the T_2 generation were selected and two lines were sown in subsequent generations from each promoter construct. In each case Mendelian segregation of the transgenes was observed, which corresponded precisely with appearance of the fruit-specific phenotypes described above. The plants remained healthy in all cases.

4.4 Discussion

Mutations in the tomato *DET1* gene were previously found to be responsible for *high pigment 2* mutant phenotypes (Mustilli et al., 1999), which are characterized by higher chlorophyll contents in immature fruits and increased carotenoids in mature fruits. Modulation of *TDET1* expression levels in transgenic plants subsequently revealed that such characteristics were due to reduced levels of *TDET1* (Chapter 3). Unwanted consequences of reduced *TDET1* levels are the severe developmental defects such as dwarfing that occur in the vegetative parts of the plant (Chapter 3), which has limited the introduction of *hp* alleles into commercial germplasm. Such phenomena are likely due to the global regulatory role of DET1 in the control of photomorphogenesis (Schäfer and Bowler, 2002). In this Chapter it is demonstrated that these collateral negative effects can be overcome by suppressing *TDET1* expression specifically in the fruits using inverted-repeat silencing constructs expressed from fruit-specific promoters.

To obtain successful fruit-specific silencing, three different fruit-specific promoters were tested (Pear et al., 1989; Santino et al., 1997). All three promoters are characterized as being expressed during the early stages of fruit development, which was considered to be necessary in order to harness the useful effects of *DET1* suppression in fruits, as inferred from the highly pigmented nature of immature fruits of *hp2* mutants. The results indeed demonstrated that each of the three promoter constructs were able to increase carotenoid concentrations to levels substantially higher than are observed in fruits from wild-type plants. Lycopene levels were generally increased by more than 200%, and sometimes by 300%, whereas β-carotene levels

were typically 300% higher than in wild-type fruits and were sometimes close to 10-fold higher.

To my knowledge, this is the first time that such dramatic simultaneous increases in the levels of both carotenoids have been observed. Previously, overexpression in tomato of bacterial genes encoding carotenoid biosynthetic enzymes were reported to result in much more moderate increases in carotenoid levels (Romer et al., 2000; Fraser et al., 2002). Furthermore, increases in one carotenoid were found to occur at the expense of the other, which was proposed to be a consequence of rate-limiting steps within the carotenoid biosynthetic pathway, or perhaps indicative of carotenoid storage capacities being already saturated within tomato fruits (Fraser et al., 2002). The results presented here demonstrate emphatically that this is not the case and reveal that manipulation of regulatory genes controlling carotenoid biosynthesis can generate enormous increases in flux through the whole pathway.

It would therefore appear likely that even greater increases in carotenoid content can be achievable through the judicious manipulation of key regulatory genes than through manipulation of genes encoding biosynthetic enzymes. This finding also has implications for increasing the provitamin A content of golden rice (Ye et al., 2000), because β-carotene levels of rice varieties engineered with genes encoding carotenoid biosynthetic enzymes are still far below recommended daily allowances for alleviation of vitamin A deficiency-related diseases (Potrykus, 2001).

Interestingly, the P119 promoter construct was more efficient than the other two promoters in increasing both lycopene and β-carotene concentrations. The major characteristic of this promoter that distinguishes it from the 2A11 and TFM7 promoters is that it is expressed throughout fruit development. Comparisons of expression patterns of these, as well as the late-expressing E8 promoter (Deikman et al., 1998) (which was previously found not to increase fruit carotenoid levels in *DET1* silencing constructs; (see Chapter 3) would suggest that inhibition of DET1 activity during early fruit development is essential for increasing mature fruit carotenoid levels, and that further increases in β-carotene levels can be achieved by the sustained suppression of DET1 activity at later stages of fruit maturation. These results suggest that individual events within the carotenoid biosynthetic pathway may occur at different times, e.g., the regulation of lycopene β-cyclase (which converts lycopene to β-

carotene) by DET1 may be most pronounced in mature fruits (Pecker et al., 1996), whereas the preceding biosynthetic steps may be more sensitive to regulation by DET1 at earlier stages. The plants containing the different promoter constructs utilized in the present study could therefore be useful tools to probe temporal differences in DET1 regulatory activities in fruits.

It is evident that inhibition of DET1 activity during the early stages of fruit development is necessary for upregulation of carotenoid biosynthesis, even though the major fruit carotenoids are synthesized much later on. Currently, there is only limited knowledge about how plastid development is regulated during fruit ripening, how they are converted into chromoplasts and how carotenoids increase within them (Hirschberg, 2001; Bramley, 2002). Because the early stages of fruit development are characterized by chloroplast biogenesis and replication, one explanation for the increased chlorophyll content of immature fruits of tomato *hp* mutants is that the *hp* mutations increase plastid compartment size within the cells of the fruit (Cookson et al., 2003). Because carotenoid biosynthesis occurs in ripening fruits within the chromoplasts, it is possible that the influence of DET1 on carotenoid biosynthesis is related to its regulation of plastid number and not directly to regulation of the carotenoid biosynthetic enzymes. Nonetheless, the differences in relative amounts of lycopene and β-carotene as a consequence of *DET1* suppression by differently expressed promoters would infer that DET1 can control directly at least some of the carotenoid biosynthetic enzymes. The transgenic plants containing the different promoter constructs generated in this study represent ideal reagents for studying the regulation of carotenoid biosynthesis during tomato fruit ripening.

It is also worth noting that fruits of *hp* mutants show increased levels of flavonoids in addition to carotenoids (Yen et al., 1997), so it is likely that this will also be found to be the case in fruits from the transgenic plants generated in this study. Future confirmation of this would further highlight the potential of the present approach for generating plant-based products with increased benefits for human health, because such substances have a range of demonstrated health benefits (Hertog et al., 1995).

The high efficiency of fruit-specific silencing of *DET1* in these experiments resulted at worst in only minor reductions in yield. This was likely a consequence of the combination of fruit-specific promoters with high-efficiency silencing constructs, i.e., made up of an aberrant

TDET1 coding sequence in an inverted-repeat configuration. The highly fruit-localized nature of *DET1* silencing indicates that silencing signals did not propagate from the fruits to the rest of the plant. This is probably because fruits are sink rather than source organs and so do not possess efficient mechanisms for transport of substances out of them. Furthermore, this result reveals that manipulation of carotenoid biosynthesis specifically within the fruits does not result in the diversion of metabolites away from other important biosynthetic pathways such as for the phytohormones gibberellic acid and abscisic acid, which would have resulted in dwarf phenotypes and delayed germination (Lindgren et al., 2003). Of significance for understanding the mechanisms of systemic transfer of gene silencing signals is that the silencing signal was not passed through the seed, because seeds derived from *TDET1*-silenced fruits gave rise to wild-type seedlings that developed like normal plants up until fruit development. A previous study also proposed that epigenetic silencing signals are not transmitted through meiosis (Mitsuhara et al., 2002).

In summary, this study shows that the judicial manipulation of a plant regulatory gene can result in dramatic increases in fruit carotenoid content with essentially no negative collateral effects on fruit yield or quality. Such transgenic strategies may prove to be more acceptable to the general public than currently utilized genetically modified crops because they are based solely on the use of plant genes. Furthermore, the combination of PTGS technology with tissue-specific expression systems is likely to generate phenotypes that cannot be achieved by conventional breeding approaches. The fruit-suppressed *TDET1* transgenic plants generated in this study provide valuable material for studying the fruit ripening process, and also can provide interesting new tomato-based products with enhanced benefits for human health.

4.5 Materials and Methods

4.5.1 Construction of Plasmids.

All expression cassettes were made by Seminis Vegetable Seeds Inc (Woodland, CA-USA) in the binary vector SVS297nos (Burgess et al., 2002), which contains *NPT II* as kanamycin resistant marker gene driven by the CaMV 35S promoter. A schematic representation of construct generation is illustrated in Figure 4.1. PCR-based cloning was used to generate

suitable restriction sites to fuse each of the fruit specific promoters with the *TDET1* gene. Promoter TFM7 (Santino et al., 1997) (GENBANK: X95261) is 2.5 kb in length and was cloned in two steps. The upstream 1 kb fragment was cloned between *Not*1 and *Spe*1 sites and the remaining 1.5 kb fragment was fused between *Spe*1 and *Nco*1 sites. Similarly, the 2.5 kb P119 promoter fragment (Santino et al., 1997; Dunsmuir and Stott, 1997) (US patent 5633440) was cloned in a single step between *Not*1 and *Nco*1 sites. The 4 kb 2A11 promoter fragment (Pear et al., 1989) (GENBANK: X13741) was cloned in three steps. An upstream 1 kb fragment was cloned between *Not*1 and *Ava*II, an internal 1.2 kb fragment was fused between *Ava*II and *Hind*III, and the remaining 1.8 kb fragment was inserted between *Hind*III and *Nco*1 sites. The *TDET1* inverted-repeat was cloned in two steps downstream of the three promoters. In all cases the *TDET1* sequence contained the *hp2* mutant allele (Mustilli et al., 1999), which was previously shown to improve the efficiency of *TDET1* gene silencing (Chapter 3). The first 150 base pairs of the *TDET1* gene were truncated and the remaining 1.5 kb of the gene was cloned between *Nco*1 and *Avr*II sites. This was done by converting codon 61 (TTT; Phe) to ATG (Met) and adding an *Nco*1 site at the new ATG, retaining the frame of translation. Finally, a 520 bp 3' fragment of the gene was cloned in a reverse orientation between *Avr*II and *Sac*1 sites to create the inverted-repeat constructs. The *TDET1* inverted-repeat was cloned downstream of each promoter to generate the constructs *P119::hp2-IR::nos*, *2A11::hp2-IR::nos*, and *TFM7::hp2-IR::nos*. All chimeric gene constructs were confirmed by DNA sequencing and each construct was transformed into *A. tumefaciens* strain ABI by electroporation.

4.5.2 Plant Transformation

To generate transgenic plants, cotyledons from two-week-old seedlings were used for protocols as described by Yoder et al. (1998). The T56 tomato genotype was used for all transformations, an indeterminate commercial processing tomato variety. Briefly, tomato seeds were sterilized using 2% commercial bleach and germinated on MSSV medium (Fillatti et al., 1987). Four-to-seven-day-old cotyledons were excised and placed on freshly prepared tobacco feeder plates, prepared by decanting 1-2 mL of tobacco suspension culture cells onto 2Z medium (Thomas and Pratt, 1981). After 48 hours the cotyledons were immersed for 5 minutes in an overnight culture of *A. tumefaciens* strain ABI diluted to an OD_{600} of 0.1. After

24 hours the explants were plated on 2Z medium containing 350mg/L carbenicillin and 100mg/L kanamycin sulphate. Excised shoots were placed onto rooting medium containing 50mg/L kanamycin. Primary transformants were screened for the presence of the transgene by PCR and were transferred to soil. Adult plants were grown under shaded greenhouse conditions (25 °C day/17 °C night) at Seminis Vegetable Seeds, Woodland, California, USA. Plants were allowed to self-pollinate for the production of seeds and homozygous lines were selected on the basis of real-time RT-PCR. Dark green immature fruit phenotypes were observed in 26 out of 39 independent lines containing the P119 promoter construct, 17 out of 39 independent lines containing the 2A11 promoter construct, and 22 out of 39 of the transgenic lines containing the TFM7 promoter construct.

4.5.3 Molecular Analyses.

RNA isolation, northern blot analysis and RT-PCR was performed as described in Chapter 3.

Real-time quantitative RT–PCR was performed in triplicate using 200 ng of total RNA extracted from both leaves and fruits of the same plant. Targets were amplified in 25 µl reaction volumes using TaqMan EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA) as described in the user manual. PCR, carried out in a 7000 sequence detection system (Applied Biosystems), consisted of an initial UNG treatment (50 °C, 2 min), followed by a reverse transcription (RT) step (60 °C, 30 min), deactivation step (95 °C, 5 min), and then 40 amplification cycles (94 °C, 20 sec; 60 °C, 1 min). For a given target, differences among tissue samples were calculated using the amplification rate and the Ct difference, and were expressed as relative mRNA levels. Two plants from two independent lines per construct were tested. Data from one representative plant per construct were used to generate figure 4.3a.

4.5.4 Biochemical Analysis of Fruits

This biochemical analysis was performed by Seminis Vegetable Seeds Inc (Woodland, CA-USA) as described in Chapter 3.

Brix (Soluble Solids) values were measured on a Bellingham & Stanley RFM-91 refractometer using filtered tomato puree. Sugars were extracted from tomato puree using ethanol (final concentration 80%). Following centrifugation, an aliquot of the extract was removed and analyzed on an HP 1050 system configured with a refractometer. The sugars were separated on a Whatman Partisil PAC column using a solvent mix of 87% acetonitrile and 13% water.

CHAPTER 5

General Discussion

In the past few years a combination of molecular genetic and biochemical studies have brought remarkable progress in identifying the critical components of light signal transduction pathways and in defining their specific roles during seedling development (Møller et al., 2002; Schafer and Bowler 2002). The vast majority of these studies have been carried out in *Arabidopsis*. Notwithstanding, studies in tomato have also revealed some important aspects, for example concerning the role of photoreceptors and light signal transduction components in the regulation of fruit ripening (Adams-Phillips et al., 2004). Notable examples have been the findings that non-lethal mutations in the tomato homologues of *Arabidopsis DET1* and *DDB1* are responsible for *high pigment* mutant phenotypes (Mustilli et al., 1999; Levin et al., 2003; Liu et al., 2004; Lieberman et al., 2004). In the current thesis, the utility of tomato has been further explored, both for examining the light-dependent phenotypes of some poorly studied mutants (Chapter 2) and for exploring the possibilities to manipulate DET1 activity for understanding its function and for the generation of tomato fruits with enhanced nutritional value (Chapters 3 and 4).

The principal findings reported in Chapter 2 were the previously unexplored phenotypes of the *high pigment* mutants, *hp1* and *hp2*, in particular the root-localized anthocyanin pigment biosynthesis, their deregulated gene expression, and their responsiveness to irradiation with blue, red and far/red broad light. Furthermore, from this study with a range of largely unexplored mutants it emerged that the *atv* and *Af* mutants display a range of light hyperresponsive phenotypes that provide sound justifications for future cloning efforts.

To facilitate gene cloning in tomato, a range of resources are currently available. The tomato is in fact the principal model for the Solanaceae, which comprise more than 3000 species, including potato, pepper, aubergine and petunia. The Solanaceae represent the third most important plant taxon economically and the most valuable in terms of vegetable crops. The tomato has become the plant of choice for genetic analysis in the Solanaceae because of its diploid inheritance, ease of seed propagation, efficient sexual hybridization, short generation time (~45-100 days), and year round growth potential in greenhouses. Moreover, more than 1,000 molecular markers have been generated, with an average genetic spacing of less than 2 cM. A recently added resource has been the National Science Foundation-sponsored development of a tomato expressed sequence tag (EST) database, containing more than 150,000 ESTs from a range of tissues (www.tigr.org/tdb/lgi/index.html). Most significantly,

an international consortium has now been created that will sequence the whole genome (estimated at 900 Mb) over the next 3-5 years (www.tigr.org/tdb/lgi/index.html).

Nonetheless, current map based cloning in tomato is usually a laborious undertaking and is dependent on the availability of reliable mapping populations and the generation of a high-resolution physical map around the mutated locus. For *atv*, such studies have been hampered in the host laboratory by the difficulty of scoring *atv* mutant phenotypes in mapping populations, most probably because of the presence of modifier genes in different genetic backgrounds that affect penetrance of the mutant phenotype, and because the mutation appears to be semi-dominant. Similarly, cloning of the *Af* mutant locus is not likely to be straightforward because of its dominant nature. Most likely therefore, these mutations could be most easily identified by candidate gene mapping approaches, in analogy with what proved most successful for the *hp1* and *hp2* mutations (Mustilli et al., 1999; Levin et al., 2003; Liu, et al., 2004). Such approaches exploit the superior information available from studies in *Arabidopsis* (Causse et al., 2004).

In the case of the *atv* and *Af* mutants, similar anthocyanin-affected mutants in *Arabidopsis* and other species are typically mutated in transcription factors (e.g., *TT2*, *AN1* and *AN2*; Nesi et al., 2001; Splet et al., 2002; Borovsky et al., 2004). Notwithstanding, it will not be straightforward to identify the mutated genes in either of these mutants because myb/myc transcription factors are present in hundreds of copies in plant genomes and because no such transcription factors have yet been identified as being involved in fruit ripening. The lack of systems to study fruit ripening in *Arabidopsis* therefore indicates that it will be difficult to identify the *ATV* and *AF* genes until the tomato genome has been fully sequenced. In the meantime, it will clearly be necessary to define a precise map position of these two mutations on the tomato genome.

Because of the lack of appropriate experimental systems to study fruit ripening in *Arabidopsis*, understanding of the role of light during fruit development has been examined most thoroughly in tomato. The influence of light on the development of climacteric fruits such as tomato is now well known, beginning with a study in the 1950s that revealed an important role for Type II phytochromes in the process (Piringer & Heinze, 1954). The available evidence suggests clearly that light has the greatest impact on pigmentation and

phytonutrient accumulation (Alba et al., 2000), and the importance of individual photoreceptors has been elegantly revealed by the identification and study of photoreceptor mutants in single, double, and triple combinations (Weller et al., 2000). For example, the loss of phyB2 in a *phyAphyB1* background results in a striking reduction of chlorophyll content in immature fruits as well as a marked increase in truss length as a result of increased distance between fruits on the inflorescence axis. The loss of *cry1* in this same background also results in a reduction of chlorophyll in immature fruits but has no effect on truss architecture (Davuluri and Bowler 2005). As further evidence for the important role of photoreceptors in fruit ripening, overexpression of oat phytochrome in tomato increased pigmentation of fruits (but also increased dwarfness) (Boylan and Quail, 1989), and ectopic overexpression of the *CRY2* gene resulted in more deeply pigmented fruits together with other growth defects associated with light hyperresponsiveness (Giliberto et al., 2005).

Conversely, the RNAi-mediated suppression of *HY5*, which encodes a transcription factor involved in the activation of light-responsive genes, resulted in reduced fruit pigmentation. In addition suppression of *LeCOP1LIKE* gene expression results in significantly higher leaf and fruit pigmentation (Liu et al., 2004). These results, together with the realization that mutations in the light signalling components DET1 and DDB1 are responsible for the *hp* mutant phenotypes (Mustilli et al., 1999; Levin et al., 2003; Liu et al., 2004), clearly indicate that light signal transduction plays an essential role during the fruit ripening process, and is perhaps as important as the plant hormone ethylene, whose role has been intensively studied by molecular genetic approaches (Giovannoni, 2001).

Chapter 3 of this thesis reports the phenotypes of transgenic tomato plants in which *DET1* gene expression was modulated. A number of independent transgenic lines were generated containing different *DET1* constructs and in all cases phenotypes were a consequence of the induction of post-transcriptional gene silencing (PTGS) of the *DET1* gene. Molecular examination of these plants indicated in all cases that the phenotypes were a result of suppression of endogenous *TDET1* expression rather than the overexpression of wild-type or dominant negative versions of *TDET1*. These results therefore confirm that loss (or reductions) of DET1 activity is responsible for the *hp2* mutant phenotype (Mustilli et al., 1999).

The results also provide some new insights into the mechanism of PTGS in higher plants. Overexpression of either mutant (*hp2* and *hp2^j*) or truncated versions of the *TDET1* gene generated the strongest silencing of *TDET1*, and caused the most severe developmental defects. Hence, it appears that expression of aberrant transgenes was much more efficient at inducing PTGS than were wild-type sequences. The fact that a sequence with a single point mutation (*hp2^j*) generated such severe phenotypes may be an indication of the impressive capacity of the plant cell's surveillance system to detect abnormal RNAs (Vaucheret and Fagard, 2001). Furthermore, the wild-type phenotypes of progeny seedlings derived from silenced plants suggested that silencing was relieved during sexual reproduction and that siRNAs did not enter the seeds. Consequently, the silencing phenotype was not inherited from generation to generation, but had to be acquired in each successive generation, as was previously reported in tobacco (Mitsuhara et al., 2002).

These results also indicated that manipulation of TDET1 levels has promising biotechnological potential, because silencing of the *TDET1* gene was shown to cause elevations in β-carotene and lycopene contents in mature fruits. However, severe developmental defects such as reduced stature, bushiness, and dwarfing were also observed. In order to overcome these collateral negative effects on plant growth and to harness the positive effects of *TDET1* gene suppression in fruits, its expression should be modulated only within the fruits. Fruit-specific *TDET1* silencing constructs were therefore generated in Chapter 4, and it was demonstrated that suppression of *TDET1* expression specifically in the fruits indeed enhanced carotenoid content, without affecting plant growth and fruit yield.

The results in Chapter 4 demonstrate that each of the three fruit-specific promoter constructs tested were able to increase both lycopene and β-carotene concentrations to levels substantially higher than are observed in fruits from wild-type plants and similar or greater than have been found in the different alleles of the *hp-2* mutants (Mustilli et al., 1999; Bino et al., 2005). Previously, overexpression in tomato of bacterial genes encoding carotenoid biosynthetic enzymes were reported to result in more moderate increases in carotenoid levels (Romer et al., 2000; Fraser et al., 2002). Furthermore, increases in one carotenoid were found to occur at the expense of others, which was proposed to be a consequence of rate-limiting steps within the carotenoid biosynthetic pathway, or perhaps indicative of carotenoid storage capacities being already saturated within tomato fruits (Fraser et al., 2002). The results in

Chapter 4 demonstrate that this is not the case and reveal that manipulation of regulatory genes controlling carotenoid biosynthesis can generate impressive increases in flux through the whole pathway.

Importantly, molecular analysis of these plants showed that *TDET1* silencing did not occur in vegetative parts and, more importantly, that it did not spread to other parts of the plant. To the best of my knowledge these results provide the first example of the tissue-specific silencing of a regulatory gene for improvement of plant nutritional value. Similar combinations of PTGS technology with tissue-specific expression systems are likely to generate other phenotypes that cannot be achieved by conventional breeding approaches in the future. Furthermore, it is likely that even greater increases in carotenoid content can be achievable through the manipulation of key regulatory genes than manipulation of genes encoding biosynthetic enzymes. This finding also has implications for increasing the provitamin A content of golden rice (Ye et al., 2000), because β-carotene levels of rice varieties engineered with genes encoding carotenoid biosynthetic enzymes are still far below recommended daily allowances for alleviation of vitamin A deficiency-related diseases (Paine et al., 2005)

These results therefore demonstrate that the judicial manipulation of photoreceptor signalling pathways can be used to improve characteristics of significant commercial interest. This example also demonstrates the utility of targeting key regulatory genes rather than genes encoding biosynthetic enzymes. In the future it will be worthwhile to examine whether other phytonutrients are also increased in the *DET1*-silenced fruits. In particular, *hp* mutant fruits have previously been shown to contain increased flavonoids as well as carotenoids (Yen et al., 1997; Bino et al., 2005). Flavonoids are hydrophilic antioxidants that complement the hydrophobic antioxidant nature of carotenoids, and tomato fruits are an important dietary source. Diets rich in flavonoids have been associated with reduced risk of coronary heart disease, certain cancers and other age-related diseases (Ross and Kasum 2002). In tomato, flavonoid levels have been elevated either by amplifying biosynthetic steps (Muir et al., 2001; Niggeweg et al., 2004) or by utilizing known flavonoid transcription factor genes (Bovy et al., 2002). Although both approaches have been successful in elevating flavonoids, carotenoid content remained unaffected in these transgenic lines. By targeting DET1 it is therefore possible that both carotenoid and flavonoid levels can be increased simultaneously, which could be of significant appeal to nutritionists and to the general public, because there is

considerable interest in the development of food products rich in vitamins, flavonoids, and carotenoids as a means to benefit human health (Cooper 2004).

Transgenic approaches offer a powerful method for improving the nutritional value of plants although there is currently a great deal of public concern about their use in contemporary agriculture, in particular those containing genes derived from organisms other than plants. The utilization in Chapter 4 of tomato promoters to control the expression of a tomato-derived transgene to silence an endogenous tomato regulatory gene to generate fruits with higher nutritional value is therefore an important achievement that may have important consequences for improving the public acceptability of genetically modified plants.

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